SCIENCE CHINA
Life Sciences

•COVER ARTICLE•

May 2020 Vol.63 No.5: 623–634
https://doi.org/10.1007/s11427-020-1657-9

A seven-gene-deleted African swine fever virus is safe and effective as a live attenuated vaccine in pigs

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Received February 20, 2020; accepted February 27, 2020; published online March 1, 2020

African swine fever (ASF) is a devastating infectious disease in swine that is severely threatening the global pig industry. An efficacious vaccine is urgently required. Here, we used the Chinese ASFV HLJ/18 as a backbone and generated a series of gene-deleted viruses. The virulence, immunogenicity, safety, and protective efficacy evaluation in specific-pathogen-free pigs, commercial pigs, and pregnant sows indicated that one virus, namely HLJ/18-7GD, which has seven genes deleted, is fully attenuated in pigs, cannot convert to the virulent strain, and provides complete protection of pigs against lethal ASFV challenge. Our study shows that HLJ/-18-7GD is a safe and effective vaccine against ASFV, and as such is expected to play an important role in controlling the spread of ASFV.

African swine fever, vaccine, safety, protective efficacy, pig

Citation: Chen, W., Zhao, D., He, X., Liu, R., Wang, Z., Zhang, X., Li, F., Shan, D., Chen, H., Zhang, J., et al. (2020). A seven-gene-deleted African swine fever virus is safe and effective as a live attenuated vaccine in pigs. Sci China Life Sci 63, 623–634. https://doi.org/10.1007/s11427-020-1657-9

INTRODUCTION

African swine fever (ASF) is a devastating infectious disease in swine and has been catastrophic for the global pig industry. The causative agent, ASF virus (ASFV), belongs to the genus *Asfivirus* within the Asfarviridae family. ASFV has complicated architecture (Wang et al., 2019; Alejo et al., 2018) and a large double-stranded DNA genome (170–193 kb) containing 151–167 genes depending on the strain. Depending on the *B646L* gene, which encodes the capsid protein p72, ASFs are divided into 24 different genotypes (Quembo et al., 2018), all of which have been detected in Africa (Quembo et al., 2018; Wade et al., 2019). Two of the ASFV genotypes have spread to other continents. The genotype I ASFV emerged in Europe in the 1950s and was eradicated from most European countries by the mid-1990s (Sánchez-Vizcaíno et al., 2013; Iglesias et al., 2017; Revilla et al., 2018). The genotype II ASFV was introduced into Georgia in 2007 and since then has become prevalent in many European countries (Iglesias et al., 2017; Pejsak et al., 2014); in 2018, the virus was transmitted to pigs in China and 10 other Asian countries (Wen et al., 2019; Ge et al., 2018; Le et al., 2019; Kim et al., 2020) (http://www.oie.int/).

Pig production in China accounts for more than 50% of the pig population worldwide; however, over 99% of the pig farms in China are small-scale farms that produce less than 500 pigs annually, and these farms often have no biosecurity measures. Although the ASF outbreaks appeared to have been controlled by the slaughtering of infected pigs, the

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ASFVs widely contaminated the environment and, therefore, continued to pose a threat to the pig industry in China. Since no vaccine is available, the resumption of pig production is unlikely to succeed. Pig production in China in September 2019 was reduced by 40% compared with that in August 2018, and the price of pork has doubled since August 2019. The situation in other Asian countries may be the similar. These facts demonstrate that ASF cannot be controlled by culling infected pigs alone, and the development and application of an efficacious vaccine is urgently needed.

Different vaccine strategies for ASF have been evaluated in the past decades. Inactivated vaccines, DNA vaccines, subunit vaccines, and adenovirus-vectored vaccines have been tested and proved to be unsuccessful (Sunwoo et al., 2019; Sánchez et al., 2019; Murgia et al., 2019; Jancovich et al., 2018; Arias et al., 2017). Some gene-deleted ASFVs have shown potential as live attenuated vaccines (Borca et al., 2020a; Reis et al., 2017; O'Donnell et al., 2017; Monteagudo et al., 2017; O'Donnell et al., 2015a; O'Donnell et al., 2015b), but it is not known if they could convert to more virulent strains during their replication in pigs. In the present study, we selected the first ASFV isolated in China, Pig/Heilongjiang/2018 (HLJ/18) (Zhao et al., 2019; Wen et al., 2019), as a backbone to develop a live attenuated vaccine.

RESULTS

Generation and virulence evaluation of ASFVs with different gene deletions

Using a DNA homologous recombination technique (Reis et al., 2017; Monteagudo et al., 2017; O'Donnell et al., 2015a), we constructed six different viruses by deleting gene segments encoding one to seven different proteins, including MGF505-1R, MGF505-2R, MGF505-3R, MGF360-12L, MGF360-13L, MGF360-14L, CD2v, 9GL, DP148R, and UK, which have previously been shown to be important for the virulence of different ASFVs (Reis et al., 2017; O'Donnell et al., 2017; Monteagudo et al., 2017; O'Donnell et al., 2015a; Zsak et al., 1998; Reis et al., 2016). The gene-deleted viruses bearing one or two of the reporter genes GFP and mCherry were purified in primary porcine alveolar macrophages (PAMs), confirmed by sequence analysis, and designated as HLJ/18-6GD (genes encoding the six proteins MGF505-1R, MGF505-2R, MGF505-3R, MGF360-12L, MGF360-13L, and MGF360-14L deleted), HLJ/18-CD2v-del (gene encoding CD2v deleted), HLJ/18-DP148R-del (DP148R gene deleted), HLJ/18-9GL&UK-del (9GL and UK genes deleted), HLJ/18-CD2v&UK-del (CD2v and UK genes deleted), and HLJ/18-7GD (genes encoding MGF505-1R, MGF505-2R, MGF505-3R, MGF360-12L, MGF360-13L, MGF360-14L, and CD2v deleted), respectively (Figure 1A).

The HLJ/18 virus is highly lethal in pigs (Zhao et al., 2019), and its 50% pig lethal dose (PLD_{50}) is 1.7 50% hemadsorbing doses (HAD_{50}) (Figure 1B). To investigate whether these gene-deleted viruses were attenuated in pigs, we intramuscularly injected groups of three to six 7-week-old specific-pathogen-free (SPF) pigs with 10^3 and 10^5 $TCID_{50}$ of the gene-deleted viruses and observed the pigs for three weeks. Body temperature and survival were monitored for 21 days post-inoculation (p.i.). As shown in Figure 1B, all of the pigs inoculated with HLJ/18-DP148R-del developed fever and died within 9 days p.i., 50%–100% of pigs inoculated with HLJ/18-CD2v-del or HLJ/18-CD2v&UK-del developed fever and 50%–75% of pigs in these groups died during the 3-week observation period, whereas all of the pigs inoculated with HLJ/18-6GD, HLJ/18-9GL&UK-del, or HLJ/18-7GD remained healthy and survived the duration of the 3-week observation period (Figure 1B, Table 1). These results indicate that HLJ/18-DP148R-del is lethal in pigs, HLJ/18-CD2v-del and HLJ/18-CD2v&UK-del are slightly attenuated in pigs, and HLJ/18-6GD, HLJ/18-9GL&UK-del, and HLJ/18-7GD are dramatically attenuated in pigs.

Protective efficacy of the attenuated ASFVs against virulent HLJ/18 challenge in pigs

To investigate whether the attenuated viruses could induce protective immunity, the HLJ/18-6GD-, HLJ/18-9GL&UK-del-, and HLJ/18-7GD-inoculated pigs were challenged intramuscularly (i.m.) with 200 PLD_{50} of HLJ/18 virus; four 10-week-old SPF pigs were challenged similarly as a control. Body temperature and survival were recorded daily for 21 days. Blood, organs, and tissues including the heart, lung, spleen, tonsil, thymus and five lymph nodes (intestinal lymph node, inguinal lymph node, submaxillary lymph node, bronchial lymph node, and gastrohepatic lymph node) were collected from the dead pigs and from the surviving pigs, which were euthanized on day 21 post-challenge (p.c.) for viral DNA quantification by qPCR. The pigs in the control and HLJ/18-9GL&UK-del-inoculated groups all developed fever and died within 12 days p.c. (Figure 2A); high copies of viral DNA were detected in the blood, organs, and tissues from these pigs (Figure 2B and C). Pigs in the HLJ/18-6GD- and HLJ/18-7GD-inoculated groups all survived the duration of the observation period (Figure 2A); viral DNA was detected in some organs or tissues of the pigs in these groups (Figure 2D and E). It is noteworthy that the viral DNA copies in the HLJ/18-7GD-inoculated groups were lower than those in the other groups. One pig in each of the 10^3 $TCID_{50}$ and 10^5 $TCID_{50}$ HLJ/18-6GD-inoculated groups developed fever that lasted for 9 and 6 days, respectively (Table 2). All four pigs in the 10^3 $TCID_{50}$ HLJ/18-7GD-inoculated group developed fever, but only one pig in the 10^5 $TCID_{50}$ HLJ/18-

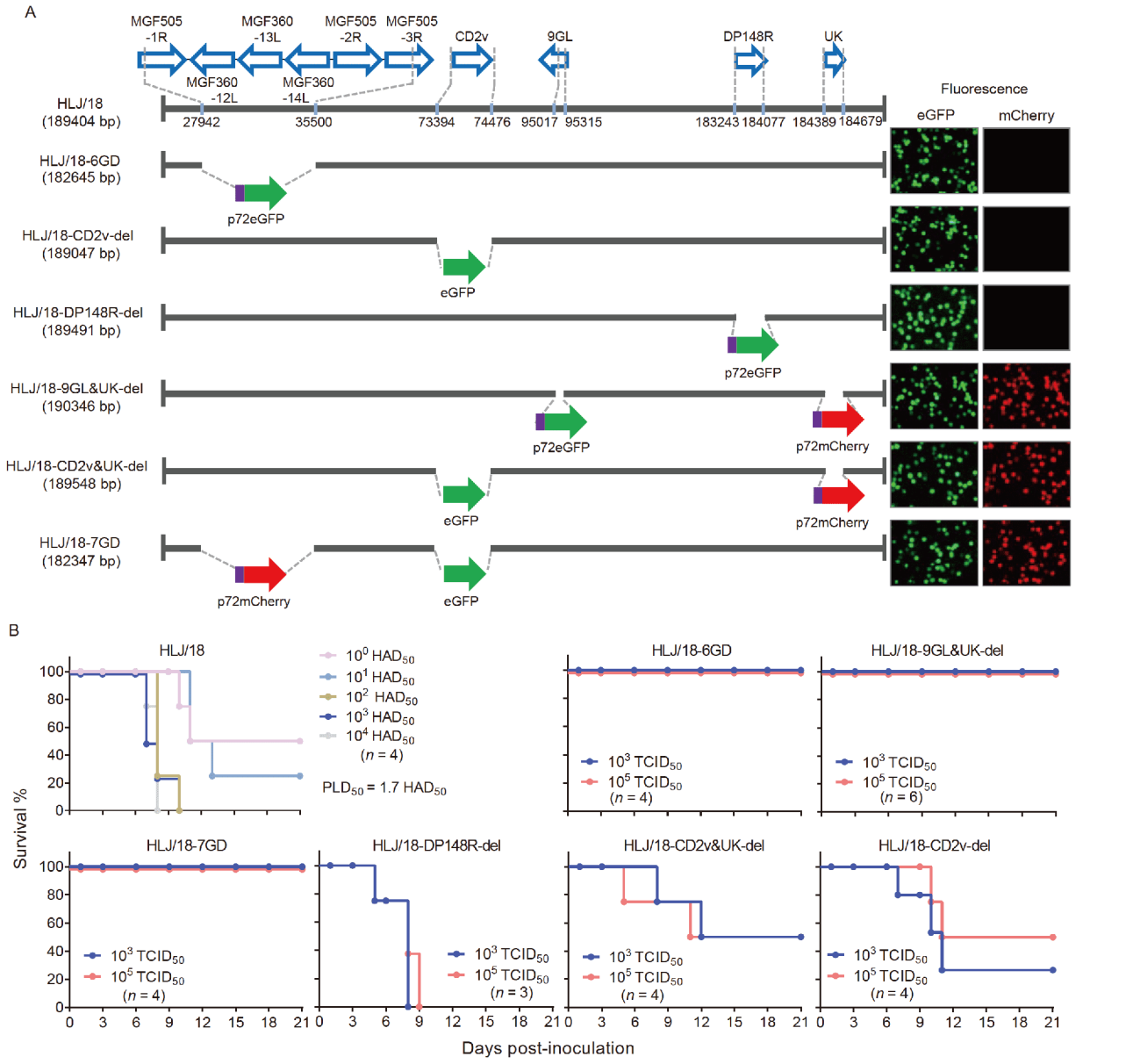

Figure 1 Generation and virulence evaluation of different gene-deleted African swine fever viruses (ASFVs). A, Schematic representation of the gene(s) and region(s) deleted in each gene-deleted ASFV. The deleted gene segments were replaced with the p72eGFP, eGFP, or p72mCherry reporter gene cassette as indicated. The virus-infected primary porcine alveolar macrophages expressing different fluorescence are shown on the right of the panel. Nucleotide positions indicating the boundaries of the deletion relative to the ASFV HLJ/18 genome are indicated. B, Survival rates of pigs inoculated with the wild-type ASFV HLJ/18 and different gene-deleted ASFVs.

7GD-inoculated group had fever for 1 day (Table 2). These results indicate that HLJ/18-9GL&UK-del does not induce any protection, whereas HLJ/18-6GD and HLJ/18-7GD are immunogenic and able to protect pigs against lethal ASFV challenge. These data also indicate that the protection induced by HLJ/18-7GD is dose-related, whereas the protection induced by HLJ/18-6GD is not.

Safety evaluation of HLJ/18-6GD and HLJ/18-7GD in pigs

The biggest concern with a live attenuated vaccine is whether the vaccine seed virus could convert to a virulent strain during replication in vaccinated animals, since two early live vaccines, which were developed by attenuating virulent strain through serial passages in cell culture or from a naturally isolated avirulent strain, converted to lethal strains and caused 10%–50% mortality during their application in farmed pigs in Portugal and Spain in the 1960's (Coggins et al., 1968; Ribeiro et al., 1963). According to the guidelines for live attenuated vaccine development in China (http://www.moa.gov.cn), a vaccine strain must be serially passed in the target animals for five passages to assess whether the animal-adapted seed strain will replicate more efficiently or

Table 1 Virulence of gene-deleted ASFVs in SPF pigs

Virus	Dose	Characteristics of fever in pigs after inoculation with each gene-deleted virus			No. of surviving pigs/total
		No. of pigs with fever/total	Duration (day)	Highest body temperature (°C)	
HLJ/18-6GD	10^3 $TCID_{50}$	0/4	/	/	4/4
	10^5 $TCID_{50}$	0/4	/	/	4/4
HLJ/18-9GL&UK-del	10^3 $TCID_{50}$	0/6	/	/	6/6
	10^5 $TCID_{50}$	0/6	/	/	6/6
HLJ/18-7GD	10^3 $TCID_{50}$	0/4	/	/	4/4
	10^5 $TCID_{50}$	0/4	/	/	4/4
HLJ/18-DP148R-del	10^3 $TCID_{50}$	3/3	4	41	0/3
	10^5 $TCID_{50}$	3/3	5	41.4	0/3
HLJ/18-CD2v&UK-del	10^3 $TCID_{50}$	2/4	3–6	41	2/4
	10^5 $TCID_{50}$	4/4	2–9	42	2/4
HLJ/18-CD2v-del	10^3 $TCID_{50}$	3/4	4–5	41.1	1/4
	10^5 $TCID_{50}$	2/4	5–6	41.4	2/4

Table 2 Protective efficacy of gene-deleted ASFVs against lethal ASFV HLJ/18 challenge in SPF pigs

Virus	Dose	Characteristics of fever in pigs after challenge with lethal HLJ/18			No. of surviving pigs/total
		No. of pigs with fever/total	Duration (day)	Highest body temperature (°C)	
Control	Not applicable	4/4	3–5	41.4	0/4
HLJ/18-9GL &UK-del	10^3 $TCID_{50}$	6/6	5–8	41.7	0/6
	10^5 $TCID_{50}$	6/6	4–8	41.6	0/6
HLJ/18-6GD	10^3 $TCID_{50}$	1/4	9	41.4	4/4
	10^5 $TCID_{50}$	1/4	6	41.9	4/4
HLJ/18-7GD	10^3 $TCID_{50}$	4/4	3–9	42	4/4
	10^5 $TCID_{50}$	1/4	1	40.7	4/4

become more lethal than the original seed strain. To this end, groups of six SPF pigs were intramuscularly inoculated with 10^7 $TCID_{50}$ of HLJ/18-6GD and HLJ/18-7GD viruses and observed for 21 days. Blood samples were collected from the pigs on days 5, 10, and 15 p.i. and spleen and lymph nodes were collected from each pig euthanized on day 21 p.i. to detect viral DNA by qPCR. In the HLJ/18-6GD-inoculated group, viral DNA was detected in the blood of five pigs and in the spleen of one pig (Table 3). However, in the HLJ/18-7GD-inoculated group, viral DNA was not detected in any samples collected (Table 3). All pigs survived the duration of the 3-week observation period.

We next passed the HLJ/18-6GD-positive blood four more passages in pigs. Blood samples were again collected on days 5, 10, and 15 p.i., and organs and tissues including the heart, liver, spleen, lung, kidney, tonsil, thymus and lymph nodes from each pig euthanized on day 21 p.i. were collected for the detection of viral DNA. We found that the HLJ/18-6GD virus progressively replicated more efficiently in pigs, as evidenced by the increased number of viral DNA copies in the blood and the tissue distributions in pigs inoculated with passage 5 virus (Figure 3A–D, Table 3). Of note, one pig in the passage 5 group for the HLJ/18-6GD virus developed severe disease and died on day 11 p.i. (Figure 3F, Table 3). The blood that was harvested on day 5 p.i. from this pig was inoculated into five more pigs, and all of these pigs (passage 6) had high viral DNA copies in their blood and organs, four of them developed fever, and one of them showed severe disease symptoms and died on day 13 p.i. (Figure 3E and F, Table 3). These results indicate that the HLJ/18-6GD virus has the potential to become more virulent during its replication in pigs.

We also blindly passed the viral DNA-negative blood of the HLJ/18-7GD-inoculated pigs for four more passages, but viral DNA was not detected in any samples collected from these pigs (Table 3). To further investigate the replication of HLJ/18-7GD in pigs, 14 seven-week-old SPF pigs were inoculated with a higher dose of $10^{7.7}$ $TCID_{50}$ of the virus, and two pigs were euthanized on days 2, 5, 8, 10, 12, 16, and 21 p.i., respectively. The blood and organs of these pigs were collected for viral DNA detection. As shown in Figure 3, viral DNA was not detected in the blood, heart, liver, spleen, lung, kidney, tonsil, or thymus of any pig, but was detected in some lymph nodes of one or two pigs that were euthanized

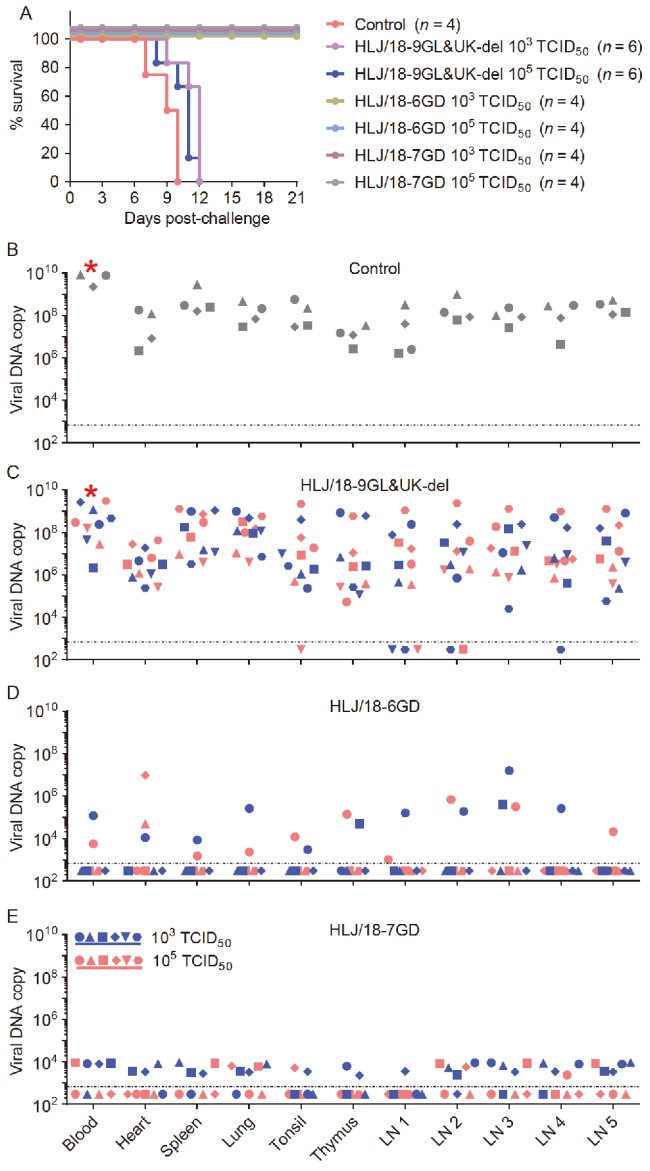

Figure 2 Protective efficacy induced by different gene-deleted ASFVs in pigs. Groups of specific-pathogen-free pigs inoculated with 10^3 or 10^5 $TCID_{50}$ of the indicated gene-deleted ASFVs were challenged intramuscularly (i.m.) with lethal ASFV HLJ/18. The indicated samples were collected from dead pigs or surviving pigs that were euthanized on day 21 post-challenge for virus DNA detection. A, Survival rates of pigs. B–E, Viral DNA detection of pigs in the control group, the HLJ/18-9GL&UK-del-inoculated groups, the HLJ/18-6GD-inoculated groups, and the HLJ/18-7GD-inoculated groups. The dashed lines indicate the lower limit of detection. The red asterisk indicates that some blood samples were not collected from pigs that died during the night.

on days 5, 8, 10, 12, and 16 p.i. (Figure 3G). The lymph nodes that had the highest viral DNA copies were homogenized and inoculated into four more pigs, and two pigs were euthanized on day 8 p.i. and the other two were euthanized on day 10 p.i.. The blood, organs, and lymph nodes of these pigs were collected for viral DNA detection. As shown in Figure 3H, viral DNA was detected in two lymph nodes of one pig that was euthanized on day 10 p.i., but was not detected in any samples collected from the other three pigs (Figure 3H). The virus was not detected in any subsequent pigs inoculated with the viral DNA-positive lymph node homogenates (data not shown). These results indicate that, after intramuscular injection, the HLJ/18-7GD virus is only maintained for a short period in certain lymph nodes of pigs, and does not appear in the blood or any other organs of pigs; therefore, HLJ/18-7GD is highly unlikely to convert to a virulent strain during its replication in pigs. Based on these safety evaluations, we selected the HLJ/18-7GD virus as a vaccine strain for ASF and performed additional studies in clean commercial pigs.

Protective efficacy of HLJ/18-7GD against virulent HLJ/18 challenge in commercial pigs

Since the protective immunity induced by a single dose of vaccine may decline in a few weeks, a second dose may be needed in actual animal disease control practice. In addition to intramuscular challenge, we also evaluated the protective effects of challenging pigs orally, as the oral route is how pigs are mainly infected with ASFV in nature. Groups of 7-week-old piglets from a local farm were transferred to a P2 facility and vaccinated once or twice (with a 3-week interval) with 10^5 $TCID_{50}$ of the vaccine and were then moved to a P4 facility for challenge at different timepoints after vaccination. Groups of four or five similar aged unvaccinated pigs were used as controls. All pigs were observed for three weeks after challenge with the lethal HLJ/18 virus. Samples, including the blood, heart, spleen, lung, tonsil, thymus, and lymph nodes, from dead pigs and surviving pigs that were euthanized on day 21 p.c. were collected for viral DNA detection by qPCR. Of note, the control pigs in all of the challenge studies died within 11 days of challenge, and high viral DNA copy numbers were detected in all of the organs tested from these pigs (Figure 4, Table 4).

As shown in Figure 4A, the pigs that were inoculated with one dose of 10^5 $TCID_{50}$ vaccine and challenged i.m. with 200 PLD_{50} of HLJ/18 on day 28 post-vaccination all survived the 3-week observation period, but viral DNA was detected in the blood, tonsil, and two lymph nodes of one of the five pigs that were euthanized at the end of the observation period (Figure 4A), indicating that HLJ/18-7GD provides similar protection in both farmed and SPF pigs.

Two groups of pigs were inoculated twice with 10^5 $TCID_{50}$ of the vaccine: one group was challenged i.m. with 200 PLD_{50} of HLJ/18 two weeks after the second dose, and the other group was challenged orally with $10^{6.5}$ HAD_{50} of HLJ/18 three weeks after the second immunization. All vaccinated pigs survived the 3-week observation period, and very low levels of viral DNA were detected in one lymph node of one pig in the i.m. challenge group (Figure 4B); viral DNA was not detected in any pigs in the orally challenged group

Table 3 Viral DNA detection from pigs that were used for the safety evaluation of the HLJ/18-6GD and HLJ/18-7GD viruses[a)]

Virus	No. of passage in pigs	Number of viral DNA-positive pigs/total number of pigs																No. of pigs with fever /total
		Blood on days post-inoculation (p.i.) (highest copy number, log10)			Samples collected from pigs euthanized on day 21 p.i. or from pigs that died before day 21 p.i.													
		Day 5	Day 10	Day 15	Blood	Heart	Liver	Spleen	Lung	Kidney	Tonsil	Thymus	LN 1	LN 2	LN 3	LN 4	LN 5	
HLJ/18-6GD	1	4/6 (6.0)	4/6 (6.1)	4/6 (6.1)	ND	ND	ND	1/6	ND	ND	ND	ND	0/6	0/6	0/6	0/6	0/6	0/6
	2	3/3 (6.6)	3/3 (7.2)	3/3 (7.1)	3/3	1/3	1/3	2/3	1/3	0/3	0/3	0/3	0/3	0/3	1/3	1/3	2/3	0/3
	3	2/3 (6.6)	3/3 (7.9)	ND	3/3	1/3	1/3	2/3	2/3	1/3	0/3	1/3	1/3	0/3	2/3	2/3	2/3	1/3
	4	3/3 (8.3)	3/3 (7.9)	3/3 (5.9)	3/3	0/3	0/3	3/3	0/3	1/3	1/3	1/3	0/3	0/3	2/3	0/3	0/3	0/3
	5*	3/3 (8.1)	3/3 (8.0)	3/3 (6.0)	3/3	3/3	2/3	3/3	3/3	2/3	2/3	3/3	2/3	1/3	2/3	1/3	1/3	2/3
	6**	4/5 (8.7)	5/5 (9.4)	4/4 (8.2)	4/4	4/5	5/5	5/5	5/5	5/5	3/5	3/5	4/5	3/5	3/5	4/5	4/5	4/5
HLJ/18-7GD	1	0/6	0/6	0/6	ND	ND	ND	0/6	ND	ND	ND	ND	0/6	0/6	0/6	0/6	0/6	0/6
	2 to 5	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12

a) Groups of six pigs were intramuscularly injected with 10^7 $TCID_{50}$ of each test virus, and their blood, organs, and tissues were collected at the indicated timepoints for viral DNA detection. Viral DNA-positive blood samples collected from the HLJ/18-6GD-inoculated pigs were used to inoculate pigs in the subsequent passage (passages 2–6). Since the viral DNA was not detected from any pigs that were inoculated with the HLJ/18-7GD virus, the pooled blood samples collected from pigs on days 5 and 10 post-inoculation were used to inoculate pigs in the subsequent passage (passages 2–5). *, one pig in this group died on day 11 post-inoculation. **, one pig in this group died on day 13 post-inoculation, and its blood sample was not collected. LN 1: intestinal lymph node; LN 2: inguinal lymph node; LN 3: submaxillary lymph node; LN 4: bronchial lymph node; LN 5: gastrohepatic lymph node. ND, not done.

(Figure 4C). These results indicate that two immunization with 10^5 $TCID_{50}$ HLJ/18-7GD could provide solid protection against lethal HLJ/18 challenge in pigs.

Commercial pigs are usually raised for at least five months. When we challenged a group of pigs that were vaccinated twice with 10^5 $TCID_{50}$ HLJ/18-7GD at the age of five months (80 days after the last vaccination), we found that 80% of them survived the challenge, but long-lasting immunity was unable to efficiently clear the challenging virus (Figure S1 in Supporting Information). The data in Figure 2 show that the protection induced by HLJ/18-7GD was dose-related, and therefore, we investigated whether a higher inoculation dose could induce better long-term protection. To this end, we inoculated a group of six pigs with one 10^6 $TCID_{50}$ dose of the vaccine and challenged the pigs at 10 weeks post-vaccination. We found that all of the vaccinated pigs remained healthy and survived the duration of the observation period, and low levels of viral DNA were detected in one lymph node of two different pigs, but not in the other four pigs (Figure 4D). These results indicate that the long-lasting immunity induced by a single dose of 10^6 $TCID_{50}$ of HLJ/18-7GD could provide solid protection against lethal ASFV challenge, and suggest that two doses of 10^6 $TCID_{50}$ of HLJ/18-7GD administrated appropriately could protect pigs for their entire lives.

Safety evaluation of HLJ/18-7GD in pregnant sows

In practice, to control ASF, sows will also need to be vaccinated. There is always the potential for vaccine inoculation to cause problems in pregnancy. We therefore investigated the safety of the vaccine in pregnant sows. Six primiparous sows at different pregnant stages were inoculated with 10^6 $TCID_{50}$ of HLJ/18-7GD: one sow was inoculated on the 35th day of pregnancy (early gestation), two sows were inoculated on the 94th day of pregnancy (late gestation), and three sows were inoculated on the 63rd day of pregnancy (mid-gestation). Two sows were left untreated as control (Table 5). The pregnant sows were monitored daily from the day of inoculation to four weeks after the piglets were delivered. All sows remained healthy after inoculation and delivered their piglets on the expected dates (Table 5), indicating that inoculation with HLJ/18-7GD will not cause disease or abortion in pregnant sows or affect the health of the piglets.

DISCUSSION

Several genes of the ASFV have been identified to be virulence related, and viruses lacking these genes have been evaluated as potential vaccines, but none of them have been fully evaluated for safety (Borca et al., 2020a; Reis et al., 2017; O'Donnell et al., 2017; Monteagudo et al., 2017; O'Donnell et al., 2015b; O'Donnell et al., 2015a). Deletion of the DP148R gene reduced the virulence of the Benin 97/1 isolate (a genotype I virus) in pigs (Reis et al., 2017), but DP148R gene deletion did not attenuate the HLJ/18 virus. Deletion of the CD2v gene highly attenuated the virulent BA71 strain (a genotype I virus) *in vivo* (Monteagudo et al., 2017), but slightly attenuated the HLJ/18 virus in the present study; however, a recent study reported that deletion of CD2v gene did not attenuate the Georgia/2010 strain (a genotype II strain) (Borca et al., 2020b). Another study reported that the ASFV Georgia/2007 (a genotype II strain)

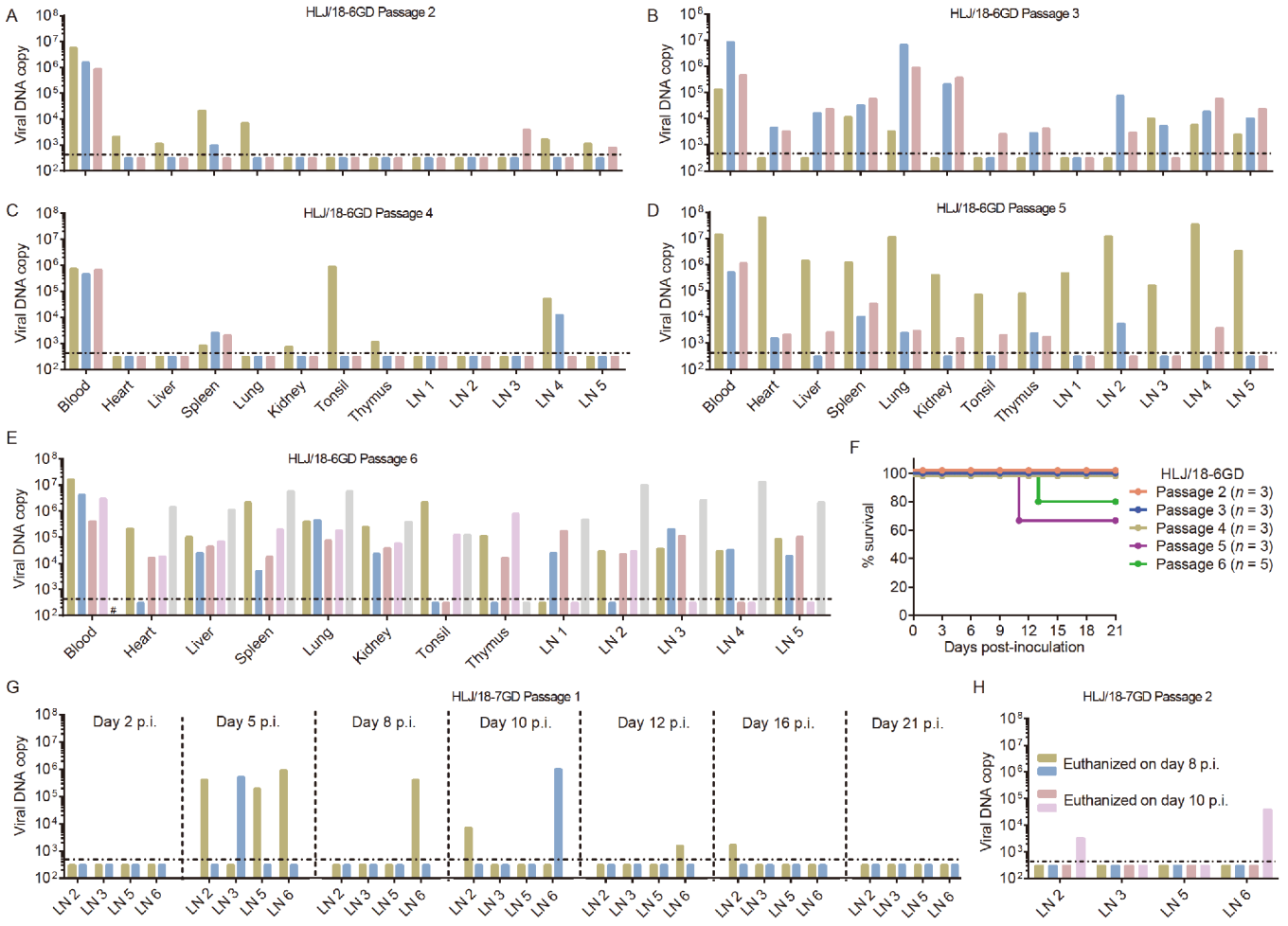

Figure 3 Safety evaluation of HLJ/18-6GD and HLJ/18-7GD in pigs. HLJ/18-6GD and HLJ/18-7GD were serially passed in pigs, and the indicated samples from the dead pigs or surviving pigs that were euthanized on day 21 post-inoculation (p.i.) were collected for viral DNA detection. The viral HLJ/18-6GD DNA copies from the 2nd to 6th passage are shown in panels A to E, respectively. The survival rate of pigs inoculated with different passages of HLJ/18-6GD is shown in panel F. Viral DNA was not detected in any samples collected from the HLJ/18-7GD initially inoculated pigs or the blindly passed pigs (Table 3). Therefore, 14 pigs were inoculated with the $10^{7.7}$ $TCID_{50}$ dose of HLJ/18-7GD and two pigs at each timepoint were euthanized on days 2, 5, 8, 10, 12, 16, and 21 p.i.. Viral DNA was detected in a few lymph nodes of some pigs (G), and in two lymph nodes of one pig after the second passage (H), but was not detected in the blood, heart, liver, spleen, lung, kidney, tonsil, thymus, or other lymph nodes of any pigs (data not shown). The dashed lines indicate the lower limit of detection. LN 1: intestinal lymph node; LN 2: inguinal lymph node; LN 3: submaxillary lymph node; LN 4: bronchial lymph node; LN 5: gastrohepatic lymph node; LN 6: mediastinal lymph node.

with the 9GL and UK genes deleted was safe and protected pigs against homologous virus challenge (O'Donnell et al., 2017); however, the HLJ/18 virus with a similar deletion (HLJ/18-9GL&UK-del) was attenuated in pigs, but did not provide any protection against homologous virus challenge. These findings suggest that the molecular basis of the virulence of ASFV may vary among strains, and that biological changes induced by gene deletion in one ASFV strain may not be similar in other strains.

Similar to the findings with ASFV Georgia/2007 and Benin 97/1 (Reis et al., 2016; O'Donnell et al., 2015a), our HLJ/18-6GD in which the MGF360 and MGF505 genes are deleted was also attenuated in pigs and protected pigs against challenge with the virulent parental virus; however, our safety evaluation data indicated that HLJ/18-6GD has a high risk converting to a virulent strain, and therefore, should not be used as a vaccine. Fortunately, for the HLJ/18-7GD virus, this risk has been eliminated by deletion of the CD2v gene.

Previous studies indicated that ASF vaccines easily lost their immunogenicity after adaptation in cell lines and could not confer protection against homologous virus challenge in pigs (Sánchez et al., 2019; Gallardo et al., 2018; Krug et al., 2015), suggesting that the ASF vaccine should be produced in primary cells, which may limit large-scale production. The porcine bone marrow (PBM) cells have been reported to support ASFV replication (Malmquist and Hay, 1960). We found that PBMs supported the growth of HLJ/18-7GD well and at least 200,000 doses of vaccine (10^6 $TCID_{50}$/dose) could be produced from PBMs derived from one SPF piglet. Moreover, we found that the HLJ/18-7GD maintains its immunogenicity after being serially passed in PBMs for six passages (data not shown). Therefore, using the primary

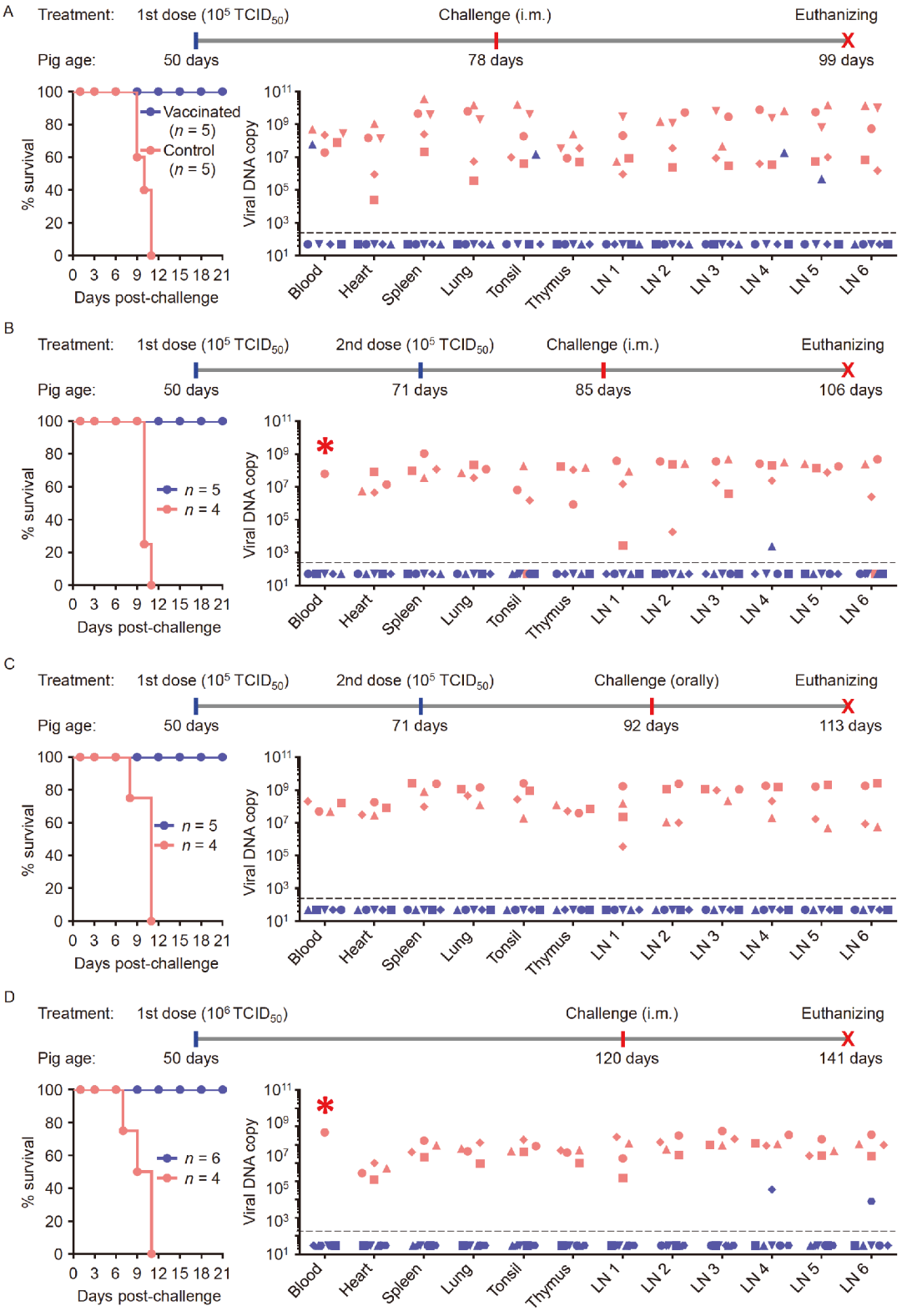

Figure 4 Vaccine efficacy of the HLJ/18-7GD in commercial pigs. Groups of clean commercial pigs were inoculated once (A, D) or twice (B, C) with 10^5 $TCID_{50}$ (A, B, C) or 10^6 $TCID_{50}$ (D) of LJL/18-7GD vaccine, and then challenged at the indicated timepoints with lethal HLJ/18 virus intramuscularly (i.m.) (A, B, D) or orally (C); similar aged unvaccinated pigs were used as controls. Pigs were observed for 21 days post-challenge and then euthanized for detection of the viral DNA of the challenging virus. The survival rates and viral DNA copies in the organs and tissues of the pigs are shown in the panels. The dashed lines indicate the lower limit of detection. The red asterisk indicates that some blood samples were not collected from pigs that died during the night. LN 1: intestinal lymph node; LN 2: inguinal lymph node; LN 3: submaxillary lymph node; LN 4: bronchial lymph node; LN 5: gastrohepatic lymph node; LN 6: mediastinal lymph node.

Table 4 Protective efficacy of HLJ/18-7GD in commercial pigs

Administration				Characteristics of fever in pigs after challenge with lethal HLJ/18			No. of surviving pigs/total
Challenge route	Challenge time (days after last vaccination)	Vaccine dosage	Group	No. of pigs with fever/total	Duration (day)	Highest body temperature (°C)	
Intramuscular	28	10^5 $TCID_{50}$, once	Vaccinated	1/5	2	40.6	5/5
			Control	5/5	4–8	42.0	0/5
Intramuscular	14	10^5 $TCID_{50}$, twice	Vaccinated	1/5	2	41.2	5/5
			Control	4/4	5–6	42.0	0/4
Oral	21	10^5 $TCID_{50}$, twice	Vaccinated	1/5	2	41.8	5/5
			Control	4/4	4–6	42.0	0/4
Intramuscular	70	10^6 $TCID_{50}$, once	Vaccinated	2/6	2	41.3	6/6
			Control	4/4	4–8	42.0	0/4

Table 5 The safety of HLJ/18-7GD in pregnant sows[a)]

Sow number	Time (days) of pregnancy of the sow			Piglet information			
	Transferred to the lab (stage of gestation)	Vaccinated	Delivered	Total number	Number of mummified fetuses	Number of stillborn piglets	Healthy rate (%)
1	89 (late)	94	112	11	0	0	100
2	89 (late)	94	114	19	1	0	94.7
3	58 (middle)	63	117	15	1	1	86.7
4	57 (middle)	62	114	18	2	0	88.9
5	57 (middle)	62	114	18	0	1	94.4
6	30 (early)	35	115	18	1	0	94.4
7	54 (middle)	NA	110	19	0	2	89.5
8	27 (early)	NA	115	14	1	1	85.7

a) All pigs were primiparous sows.

PBMs for large-scale production of HLJ/18-7GD is feasible and cost-effective.

In summary, we used the Chinese ASFV HLJ/18 as a backbone to generate six viruses bearing different gene deletions, and found that HLJ/18-7GD, which has seven genes deleted, is fully attenuated in pigs, has a low risk of converting to a virulent strain, and could induce solid protection in pigs against lethal ASFV challenge. HLJ/-18-7GD has been fully evaluated and proven to be safe and effective against ASFV. We therefore expect that this vaccine will play an important role in the control of ASFV.

MATERIALS AND METHODS

Ethics statements

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The protocols were approved by the Committee on the Ethics of Animal Experiments of the Harbin Veterinary Research Institute (HVRI) of the Chinese Academy of Agricultural Sciences (CAAS) and the Animal Ethics Committee of Heilongjiang Province, China.

Biosafety statement and facility

All experiments with live ASF viruses were conducted within the enhanced biosafety level 3 (P3+) and level 4 (P4) facilities in the HVRI of the CAAS approved by the Ministry of Agriculture and Rural Affairs and China National Accreditation Service for Conformity Assessment.

Cell culture, viruses, and pigs

Primary porcine alveolar macrophages (PAMs) were collected from 30–40-day-old specific-pathogen-free (SPF) pigs, and the cells were maintained in 10% FBS RPMI 1640 medium (Thermo Scientific, USA) at 37°C with 5% CO_2. Peripheral blood mononuclear cells (PBMCs) were prepared from EDTA-treated swine blood by using a pig PBMC isolation kit (TBD Sciences, China). Porcine bone marrow (PBM) cells were collected as described previously

(Malmquist and Hay, 1960). ASFV Pig/Heilongjiang/2018 (HLJ/18) was isolated from field samples in China as described previously (Zhao et al., 2019). The HLJ/18 stock used for challenging studies was prepared from the defibrinated blood of virus-inoculated SPF pigs and titrated in PAMs.

SPF Large White and Landrace-crossed pigs were obtained from the Laboratory Animal Center of the HVRI. Clean commercial pigs and pregnant sows were obtained from local farms where the animals were tested to ensure they were negative for porcine respiratory and reproductive syndrome (PRRS), classical swine fever (CSF), ASFV, and pseudorabies virus (PRV).

Generation of gene-deleted ASFVs

Gene-deleted ASFVs were generated by homologous recombination between the parental ASFV HLJ/18 genome and recombination transfer vectors by transfection and infection procedures. Recombinant transfer vectors containing the flanking genomic regions of the targeted genes, mapping approximately 500–1,000 bp to the left and right of these deleted parts, and the reporter gene cassettes containing the reporter genes of eGFP or mCherry with the ASFV p72 late gene promoter or CD2v gene promoter were used as shown in Figure 1. Recombinant transfer vectors were constructed by using fusing PCR and the Gibson Assembly technique (Invitrogen Life Sciences, USA). PAMs were transfected with the constructed transfer vectors by using the TransIT-LT1 transfection reagent (Mirus Bio, Madison, WI, USA) and then infected with HLJ/18 at 24 h post-transfection as described previously with minor modifications (Reis et al., 2017; O'Donnell et al., 2015a; Monteagudo et al., 2017; O'Donnell et al., 2015b). These constructions created the expected deletions in the viral genome as shown in Figure 1. HLJ/18-9GL&UK-del, HLJ/18-CD2v&UK-del, and HLJ/18-7GD with two deleted parts of the viral genome were constructed by using two successive homologous-recombination procedures. The resulting virus from each homologous recombination event was purified by successively picking fluorescent plaques combined with limited dilution on monolayers of PAMs. The virus obtained from the last round of purification was amplified in PAMs to make a virus stock. To ensure the absence of parental HLJ/18 and the desired deletions in each recombinant genome, virus DNA was extracted from the virus stock and confirmed by PCR and sequencing.

Virus titration

The wild-type HLJ/18 virus was quantified by using the hemadsorption (HAD) assay as described previously (Malmquist and Hay, 1960) with minor modifications. PBMCs were seeded in 96-well plates. The samples were then added to the plates and titrated in triplicate using 10-fold serial dilutions. HAD was determined on day 7 post-inoculation (p.i.), and 50% HAD doses (HAD_{50}) were calculated by using the method of Reed and Muench (Reed and Muench, 1938).

Samples from the gene-deleted ASFV-infected pigs or cell supernatants were quantified by testing their 50% tissue culture infectious dose ($TCID_{50}$). PBMCs were seeded into 96-well plates, and three days later 10-fold serially diluted samples were added into each well in triplicate. After seven days of culture, the fluorescent protein expression was assessed by using fluorescence microscopy (Axio Observer.Z1, Zeiss, Germany). $TCID_{50}$ was calculated by using the method of Reed and Muench.

qPCR

ASFV genomic DNA was extracted from cell supernatants, tissue homogenate, or EDTA-treated whole peripheral blood by using GenElute™ Mammalian Genomic DNA Miniprep Kits (Sigma Aldrich, USA). qPCR was carried out on a QuantStudio 5 system (Applied Biosystems, USA) according to the OIE-recommended procedure.

Pig studies

To determine the 50% pig lethal dose (PLD_{50}) of HLJ/18, five groups of four 50-day-old SPF pigs were inoculated intramuscularly (i.m.) with 10-fold serial dilutions containing 10^0 to 10^4 HAD_{50} of virus in a 1 mL volume. The pigs were monitored daily for 21 days for temperature and mortality. The PLD_{50} was calculated by using the method of Reed and Muench.

To evaluate the virulence of the gene-deleted ASFVs in pigs, groups of 50-day-old SPF pigs were i.m. inoculated with 10^3 $TCID_{50}$ and 10^5 $TCID_{50}$ of each test virus, and the pigs were monitored daily for 21 days for temperature and mortality.

To evaluate the protective efficacy of the gene-deleted virus against virulent ASFV challenge, groups of pigs were i.m. inoculated once or twice with the indicated dose of gene-deleted ASFV, and were then challenged with lethal HLJ/18 virus at the indicated timepoint. Similar aged untreated pigs were challenged as controls. The pigs were monitored daily for 21 days post-challenge (p.c.) for clinical signs and mortality. Blood, organs, and tissues including the heart, liver, spleen, lung, kidney, tonsil, thymus, and lymph nodes from the dead pigs or surviving pigs that were euthanized at the end of the observation period were collected for the detection of viral DNA by using qPCR as recommended by the OIE

(King et al., 2003).

Safety evaluation of the gene-deleted ASFVs as live-attenuated vaccines in pigs

HLJ/18-6GD and HLJ/18-7GD were evaluated for safety in SPF piglets by following the recommendations of the OIE described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019 (https://www.oie.int/standard-setting/terrestrial-manual/access-online/) and the guidelines for live attenuated vaccine development in China (http://www.moa.gov.cn/nybgb/2006/dbq/201806/t20180616_6152332.htm) with minor modifications. Briefly, groups of six pigs were intramuscularly inoculated with 10^7 $TCID_{50}$ of test virus. Blood samples collected from pigs on days 5, 10, and 15 p.i. and organs and tissues collected from pigs that were euthanized on day 21 p.i. were used for viral DNA detection. Viral DNA-positive blood samples collected from the HLJ/18-6GD-inoculated pigs were used to inoculate pigs for the subsequent passage. Since viral DNA was not detected from any pigs that were inoculated with the HLJ/18-7GD virus, the pooled blood samples collected from pigs on days 5 and 10 p.i. were used to inoculate pigs for the subsequent passage.

To further evaluate the safety of HLJ/18-7GD in pigs, 14 50-day-old SPF pigs were i.m. inoculated with the $10^{7.7}$ $TCID_{50}$ of HLJ/18-7GD, and then two pigs were euthanized on days 2, 5, 8, 10, 12, 16, and 21 p.i., respectively, and their samples, including the blood, heart, liver, spleen, kidney, tonsil, thymus, and six lymph nodes (intestinal lymph node, inguinal lymph node, bronchial lymph node, submaxillary lymph node, gastrohepatic lymph node, and mediastinal lymph node) were collected for viral DNA detection by qPCR. The viral-positive organs or samples were collected, homogenized, and used to inoculate subsequent pigs. The pigs after the third passage were viral DNA-negative; their lymph nodes were pooled, homogenized, and used to inoculate four SPF pigs for one more passage (the fourth passage). The pigs were monitored daily for temperature and clinical signs for 21 days, and then were euthanized to collect blood and the indicated tissues and lymph nodes for virus detection.

The safety of HLJ/18-7GD in pregnant sows

To determine the safety of HLJ/18-7GD in pregnant sows, eight primiparous sows at different stages of gestation were purchased from a local pig farm. One sow of early gestation (the 35th day of pregnancy), two sows of late gestation (the 94th day of pregnancy), and three sows of mid-gestation (the 63rd day of pregnancy) were i.m. inoculated with 10^6 $TCID_{50}$ of HLJ/18-7GD. Two sows of early gestation were untreated as controls. All sows were monitored daily for temperature and clinical signs until four weeks after the piglets were delivered.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

Acknowledgements *We thank Susan Watson for editing the manuscript. This work was supported by the National Key R&D Program of China (2018YFC1200601), Applied Technology Research and Development Project of Heilongjiang Province (GA19B301), Key-Area Research and Development Program of Guangdong Province (2019B020211004), and the grant from the State Key Laboratory of Veterinary Biotechnology Program (SKLVBP201801).*

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SUPPORTING INFORMATION

Figure S1 Protective efficacy of long-lasting immunity induced by HLJ/18-7GD in commercial pigs.

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