Assessment of the Safety and Efficacy of an Attenuated Live Vaccine Based on Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus

Xiuling Yu, Zhi Zhou, Zhen Cao, Jiajun Wu, Zhongqiu Zhang, Baiwan Xu, Chuanbin Wang, Dongmei Hu, Xiaoyu Deng, Wei Han, Xiaoxue Gu, Shuo Zhang, Xiaoxia Li, Baoyue Wang, Xinyan Zhai, Kegong Tian

OIE Porcine Reproductive and Respiratory Syndrome Reference Laboratory, China Animal Disease Control Center, Beijing, China; National Research Center for Veterinary Medicine, Luoyang, Henan Province, China

The safety and efficacy of the JXA1–R vaccine, an attenuated strain of highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV), were examined using an intramuscular challenge model in piglets. The JXA1–R vaccine was obtained by passing HP-PRRSV JXA1 through Marc-145 cells (82nd passage). Genomic sequence comparisons showed that strain JXA1–R and its parental strain, JXA1, differ by 47 amino acids, and most of these differences are scattered throughout the PRRSV genome. Four-week-old PRRSV-free piglets were inoculated intramuscularly with JXA1–R vaccine (10^3.0, 10^4.0, 10^5.0, 10^6.0, and 10^7.0 50% tissue culture infective doses [TCID50]/ml for groups 1 to 5, respectively) and then challenged intramuscularly with the 5th passage virus of JXA1 virus (JXA1-F5, 3 ml × 10^4.5 TCID50/ml) 28 days after inoculation. The humoral immune response, swine growth, clinical signs, and differential organ lesions were monitored. The results showed that all vaccinated piglets had a perceptible humoral immune response to vaccination after day 7, which then promptly increased, almost reaching the maximum sample/positive (S/P) ratio value at 28 days postimmunization. Viremia detection indicated that the viral replication levels of the challenge virus in the immunized groups (immunization doses ≥10^4.9/ml) were significantly lower than that of the virus–challenged unvaccinated control group. Piglets in groups 2 to 5 were effectively protected against lethal HP-PRRSV infection and did not show any obvious changes in body temperature or clinical signs of disease at any point during the experiment. However, two of five piglets in group 1 showed mild pathological lesions and transitory high fever. These results suggest that JXA1–R (TCID50/ml ≥10^4.9) is sufficiently attenuated and can provide effective protection against the lethal wild-type HP-PRRSV.

Porcine reproductive and respiratory syndrome (PRRS) was first discovered in the United States in 1987 (1, 2). It is characterized by reproductive failure in pregnant sows and respiratory disorder in growing swine. PRRS has spread through most of the world’s swine-producing regions and has caused substantial economic losses to the swine industry worldwide.

PRRS virus (PRRSV) is the causative agent of PRRS. It is a single-stranded, positive-sense RNA virus belonging to the family Arteriviridae, order Nidovirales (3, 4). The viral genome is approximately 15 kb in size and contains 10 open reading frames (ORFs), designated ORF1a, ORF1b, ORF2a, ORF2b, ORF3, ORF4, ORF5a, ORF5, ORF6, and ORF7 (5–8). Among these ORFs, ORF1a encodes 9 nonstructural proteins (NSPs), including NSP1α, NSP1β, and NSP2 to NSP8; ORF1b encodes NSP9 to NSP12. ORF1a and ORF1b encode the viral nonstructural proteins, which are involved in viral replication and transcription. ORF2a, ORF2b, and ORF3 to 7 encode the viral structural proteins GP2, E, GP3, GP4, GP5a, M, and N, respectively (5–8). The ORF5a protein is a novel structural protein in PRRSV, which is encoded by an alternate version of the ORF5 protein present in all arteriviruses (7, 8).

In June 2006, a highly pathogenic strain of PRRSV (HP-PRRSV) with a unique 30-amino-acid deletion in its Nsp2 coding region was isolated from diseased swine in China. Infections were characterized by high fever, high morbidity (50% to 100%), and high mortality (20% to 100%) (9, 10). Since then, HP-PRRSV has been the major epidemic viral strain in pigs in China, causing enormous economic losses. Subsequently, HP-PRRSV was detected in countries neighboring China, including Vietnam and Laos (11, 12).

Two types of commercial vaccines, modified live-attenuated vaccines (MLVs) and inactivated vaccines, are currently available against PRRSV (13, 14). Inoculation with vaccines (especially live attenuated vaccines) can provide protection against PRRSV infections and prevent the spread of this disease. Currently, many modified live-attenuated PRRSV vaccines, such as Ingelvac ATP, RespPRRS/Reprol ATP, RespPRRS MLV, and CH-1R, have been successfully employed (15). All of the vaccines against PRRSV are generally effective against homologous strains but

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Address correspondence to Xinyan Zhai, zhaixy2010@sina.cn, or Kegong Tian, tiankg@263.net.

Xiuuling Yu and Zhi Zhou contributed equally to this study.

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TABLE 1 Nucleotide and amino acid changes in the 12 different mutant passages

| ORF and NSP | Position | JXA1 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 60 | 70 | 80 | 82 | 90 | 100 |
|------------|----------|------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Nucleotide | ORF1a    |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|            | NSP1B    |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|            |          |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|            | NSP2     |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|            |          |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|            | ORF1b    |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|            | NSP9     |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|            | ORF10    |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|            | NSP11    |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|            | ORF2     |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|            | ORF3     |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|            | ORF4     |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|            | ORF5     |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| Amino acid | ORF1a    |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|            | NSP1B    |      |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |

(Continued on following page)
show varying and sometimes no effectiveness against heterologous strains (13–15). In addition, acute PRRS-like disease and atypical PRRS, clinical consequences of PRRS characterized by abortion and high mortality in pregnant gilts, were reported for MLV-vaccinated pigs (16, 17). Notably, the HP-PRRSV strain emerged and rapidly became prevalent in China and other countries and severely affected their pork industries (11, 12). Therefore, it is imperative to develop safe and effective vaccines against HP-PRRSV.

In a previous study, we reported the outbreak of pig high fever syndrome in 2006. This syndrome is caused by the current epidemic strain of HP-PRRSV, called JXA1. We then established pathogenicity assays to characterize this strain (9, 18). In this study, JXA1-R, a genetically stable, live attenuated vaccine strain against HP-PRRSV, was obtained by sequential passage of the HP-PRRSV JXA1 strain through Marc-145 cells (82nd passage). This vaccine provides safe and effective protection against a lethal HP-PRRSV JXA1-F5 challenge and can serve as an adequate vaccine against HP-PRRSV infection in herds.

**MATERIALS AND METHODS**

Serial passage of HP-PRRSV and titer measurement. The HP-PRRSV JXA1 strain (GenBank accession no. EF112445) was isolated from dead pigs in the Jiangxi Province of China by the China Animal Disease Control Center (CADC) in 2006 (18). Marc-145 cells, subclones of an African green monkey kidney epithelial cell line (MA-104), were used for viral growth.

**TABLE 1 (Continued)**

| ORF and NSP | Position | JXA1 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 60 | 70 | 80 | 90 | 100 |
|------------|----------|------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| NSP2       | 636      | T    | T  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  |
| 679        | V        | V    | V  | E  | E  | E  | E  | E  | E  | E  | E  | E  | E  | E  | E  | E  |
| 686        | D        | D    | D  | D  | D  | D  | D  | D  | D  | D  | D  | D  | D  | D  | D  | D  |
| 682        | A        | T    | T  | T  | T  | T  | T  | T  | T  | T  | T  | T  | T  | T  | T  | T  |
| 958        | A        | A    | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  |
| 981        | G        | R    | R  | R  | R  | R  | R  | R  | R  | R  | R  | R  | R  | R  | R  | R  |
| 987        | E        | G    | G  | G  | G  | G  | G  | G  | G  | G  | G  | G  | G  | G  | G  | G  |
| 1062       | R        | R    | M  | M  | M  | M  | M  | M  | M  | M  | M  | M  | M  | M  | M  | M  |
| 1128       | V        | A    | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  |
| 1155       | I        | I    | I  | I  | I  | I  | I  | I  | I  | I  | I  | M  | M  | M  | M  | M  |
| 1179       | G        | D    | D  | D  | D  | D  | D  | D  | D  | D  | D  | D  | D  | D  | D  | D  |
| 1392       | S        | L    | L  | L  | L  | L  | L  | L  | L  | L  | L  | L  | L  | L  | L  | L  |
| NSP3       | 1561     | R    | C  | C  | C  | C  | C  | C  | C  | C  | C  | C  | C  | C  | C  | C  |
| 1629       | E        | E    | E  | E  | E  | E  | E  | E  | E  | E  | E  | D  | D  | D  | D  | D  |
| NSP7       | 2331     | N    | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  |
| ORF1b      | 10       | L    | L  | L  | L  | L  | F  | F  | F  | F  | F  | F  | F  | F  | F  | F  |
| 23         | N        | S    | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  |
| 50         | Y        | Y    | Y  | Y  | Y  | Y  | Y  | Y  | Y  | F  | F  | F  | F  | F  | F  | F  |
| 118        | I        | I    | I  | I  | I  | I  | I  | I  | I  | I  | I  | I  | V  | V  | V  | V  |
| 168        | M        | M    | M  | M  | M  | M  | T  | T  | T  | T  | T  | T  | T  | T  | T  | T  |
| 251        | F        | F    | F  | F  | F  | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  |
| 12         | F        | F    | F  | F  | F  | L  | L  | L  | L  | L  | L  | L  | L  | L  | L  | L  |
| 79         | H        | H    | H  | H  | H  | H  | H  | H  | H  | H  | H  | H  | H  | H  | H  | H  |
| 225        | T        | T    | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  |
| 248        | F        | F    | F  | V  | V  | V  | V  | V  | V  | V  | V  | V  | V  | V  | V  | V  |
| 43         | D        | D    | G  | G  | G  | G  | G  | G  | G  | G  | G  | G  | G  | G  | G  | G  |
| 66         | I        | I    | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  | S  |
| 132        | S        | S    | T  | T  | T  | T  | T  | T  | T  | T  | T  | T  | T  | T  | T  | T  |
| 172        | F        | F    | F  | F  | F  | F  | V  | V  | V  | V  | V  | V  | V  | V  | V  | V  |
| 173        | P        | A    | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  | A  |
| 174        | S        | I    | I  | I  | I  | I  | I  | I  | I  | I  | I  | I  | I  | I  | I  | I  |
| 164        | G        | G    | G  | G  | G  | G  | G  | R  | R  | R  | R  | R  | R  | R  | R  | R  |

*a Boldface type indicates mutations of the nucleotides and amino acids of the JXA1-R strain.*
propagation and titration. The virus was serially passaged through Marc-145 cells using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and incubated at 37°C with 5% CO₂. When ~80% of the virus-infected Marc-145 cells showed a cytopathic effect (CPE), the virus was harvested using freeze-thaw techniques. To isolate the virus, plaque purification was performed after every five passages. The titers of different passages of HP-PRRSV JXA1 were measured by seeding Marc-145 cells into 96-well cell culture plates 1 day before infection. The 50% tissue culture infective dose (TCID₅₀) was calculated according to the Reed-Muench method (19).

RNA isolation and genome sequencing. Passages of JXA1, F10, F15, F20, F25, F30, F35, F40, F45, F50, F60, F70, F80, F82, F90, and F100 strains were subjected to whole-genome sequence determination. Viral RNA was extracted using a QiaGen RNeasy mini kit (Qiagen, Germany) according to the manufacturer’s instructions. Eighteen pairs of PCR primers were used to amplify 18 overlapping cDNA fragments to cover the complete genome of the different JXA1 strains (18). Reverse transcription PCR (RT-PCR) was performed using SuperScript III reverse transcriptase (Invitrogen, USA) for reverse transcription and PfuUltra high-fidelity DNA polymerase (Stratagene, USA) for PCR amplification. Each fragment was amplified at least three times. The amplicons were purified with an E.Z.N.A. gel extraction kit (Omega, USA) and cloned into pGEM-T Easy vector systems (Promega, USA). Recombinant clones were sequenced with an automatic DNA sequencer (ABI, USA) and spliced artificially. Clustal X 1.83 and DNASTAR software were used to analyze the sequences.

Animal studies and clinical examinations. In vivo studies were carried out at Guangdong Dahuanong Animal Health Produce Co., Ltd. Thirty-five healthy 4-week-old piglets were obtained from a PRRSV-negative farm and divided randomly into seven groups (five piglets per group). The piglets in groups 1 to 5 were immunized intramuscularly with the passage 82 (JXA1-R) harvest of the JXA1 strain at doses of 1 ml per piglet. The challenge control (group 6) and strict negative control (group 7) groups received only dilution medium. Twenty-eight days after vaccination, each piglet (except negative-control piglets from group 7) was challenged intramuscularly with 3 ml (1 × 10⁴.₅ TCID₅₀/ml) of strain JXA1-F5. The pathogenicity of strain JXA1-F5 was confirmed by our research group as described previously (9). The different treatment groups were kept in separate rooms, and the piglets were monitored for 49 consecutively days. Every day from the day of immunization to 49 days postimmunization (DPI), the clinical observations of each animal were recorded. These clinical parameters included fever, coughing, shivering, diarrhea, and changes in appetite. In addition, animals were weighed on the day of immunization (day 0), at challenge (day 28), and at necropsy (day 49) for assessments of average daily weight gain. All the animals were euthanized 21 days after the challenge, and tissue samples were collected. Protocols of all animal experiments performed in this study were approved by the CADC ethics committee (permit no. CADC-AEC-2009002).

Viremia detection by virus isolation and quantitative real-time RT-PCR analysis. To determine if the piglets were viremic during vaccination and challenge, virus isolation was performed on all serum samples from all collection days. Briefly, sera were inoculated separately into Marc-145 cells, and the CPEs were observed microscopically for 5 days. The percentage of CPE-positive wells was calculated for each group. To determine the duration of viremia of the JXA1-R vaccine strain, serum samples collected on DPI 0, 3, 5, 7, 14, 21, 28, 35, 42, and 49 were used to detect the RNA copy number of PRRSV by TaqMan-based quantitative real-time RT-PCR analysis.

Serology analysis. Serum samples were collected at 0, 3, 5, 7, 14, 21, 28, 35, 42, and 49 DPI. Antibodies specific to PRRSV were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Idexx Laboratories, Inc., Westbrook, ME, USA) according to the manufacturer’s instructions. Results of the ELISA were considered positive if the sample-to-positive (S/P) ratios were ≥0.4.

FIG 1 Mean anti-PRRSV antibody levels. Dashed line, threshold value above which titers were considered positive for anti-PRRSV antibodies.

TABLE 2 Development of viremia in infected piglets

| Group | JXA1-R vaccination dosage (TCID₅₀/ml) | 0, serum | 3, serum | 5, serum | 7, serum | 14, serum | 21, serum | 28*, serum | 35, serum | 42, serum | 49, Serum | Lung |
|-------|------------------------------------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|------|
| 1     | 3.0                                      | 0        | 1        | 2        | 4        | 3        | 2        | 1        | 4        | 2        | 1      | 2    |
| 2     | 4.0                                      | 0        | 2        | 5        | 5        | 5        | 2        | 1        | 2        | 0        | 0      | 0    |
| 3     | 5.0                                      | 0        | 2        | 5        | 5        | 5        | 2        | 0        | 0        | 0        | 0      | 0    |
| 4     | 6.0                                      | 0        | 3        | 5        | 5        | 5        | 2        | 2        | 1        | 0        | 0      | 0    |
| 5     | 7.0                                      | 0        | 3        | 5        | 5        | 5        | 3        | 0        | 0        | 0        | 0      | 0    |
| 6     | Challenge control                        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0      | 0    |
| Negative control                        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0        | 0      | 0    |

* At 28 DPI, all the piglets were challenged with 3 × 10⁴.₅ TCID₅₀/ml of HP-PRRSV JXA1-F5.
PCR. In brief, multiple-sequence alignment was carried out using 15 differential passages of JXA1 strains. Relative to the JXA1 strain, the JXA1-R strain contained two steady nucleotide mutations at sites 3572 and 3573 of the Nsp2 gene (Table 1). After JXA1-R inoculation, nucleotide mutations remained genetically stable, as determined by sequence analysis (data not shown). According to this specific nucleotide mutant, the real-time RT-PCR assay using minor groove binder (MGB) probes for rapid detection of the JXA1-R PRRSV strain in virus-infected cell cultures and clinical samples was established. The primers for the detection of JXA1-R were a forward primer at position 3513 to 3528 (5′-TCCACGCATCCTCGGG-3′) and reverse primer at position 3630 to 3649 (5′-TGCTCTCGTCAGACTCCCGT-3′). The sequence of the TaqMan probe was 5′-CCTCGGCTCCCTCCA-MGB-3′ at position 3566 to 3580. The RT-PCR assay using MGB probes for identifying the JXA1 HP-PRRSV strain was designed in the gene segment encoding NSP2 protein as described previously (20). For quantitation, the standard curve was generated as described previously (20). Each serum sample was checked in triplicate, and the mean RNA copy per milliliter of the differential samples was calculated using these values.

**Histological observations.** To evaluate histological lesions in the tissues of the different treatment groups, hematoxylin and eosin (H&E) staining was used to evaluate a series of collected specimens, including samples from the lungs, tonsils, lymph nodes, heart, liver, spleen, kidneys, brain, stomach, and intestine jejunum. These samples were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, and stained using Harris’s H&E (21, 22).

**Statistical analysis.** Normally distributed data are shown as mean ± standard deviation (SD) and were analyzed using one-way analysis of variance and the Student-Newman-Keuls post hoc test.

**RESULTS**

Amino acid mutations of different JXA1 passages. RNA was extracted from different JXA1 passages, and RT-PCR was performed as described previously (18). Compared to the parenteral virus JXA1, there were a total of 108 nucleotide changes at the 100th passage. Among these mutations were 2 nucleotide changes (at position 116[T-C] and 124[A-G]) in the 3′-untranslated region.
No nucleotide mutations were observed in the 5'UTR of any of the virus passages. Other nucleotide mutations were observed in nonstructural and structural coding regions, 50 of which were missense mutations, causing a change of 47 amino acids in ORFs 1a, 1b, 2, 3, 4, and 5 (Table 1). All of these mutations were observed at the 80th passage. No nucleotide or amino acid changes were observed from passages 80 through 100 (Table 1).

**Immunogenicity of JXA1-R and parental viruses in piglets.** The serum samples were collected from all pigs at 0, 3, 5, 7, 14, 21, 28, 35, 42, and 49 DPI. These samples were assayed for anti-PRRSV antibody with ELISA, and the S/P ratio was averaged for each group (Fig. 1). All animals were seronegative to PRRSV within 7 DPI. The antibody response in the ELISA showed that the piglets in groups 2 to 5 had seroconverted on DPI 14, while three of the five piglets in group 1 had seroconverted by DPI 14. The remaining piglets had seroconverted by DPI 21. No PRRSV-specific antibodies were detected in group 6 (challenge control) or 7 (negative control) during the 28-day postimmunization period. On day 28, the piglets in groups 1 to 6 were challenged with the 5th passage of JXA1 virus. Mean S/P ratios of all virus-treated groups were higher than those of the challenge control group (Student’s t-test, \( P < 0.05 \)) (1–5). In the challenge control group, only one piglet became seropositive on DPI 42 (14 days postchallenge). The other piglets had died by then. Swine in the negative control group remained seronegative throughout the study (Fig. 1).

**Viral isolation.** After JXA1-R inoculation, viral isolation analysis showed that one of the animals in group 1, two of the animals in groups 2 and 3 (four total), and three of the animals in groups 4 and 5 (six total) were viremic by DPI 3. On DPI 28, one of the piglets in groups 1 and 2 (two total) and two of the piglets in group 4 were PRRSV positive (Table 2). Following challenge, two of the piglets in group 2 and one piglet in group 4 were viremic on DPI 35. Four of the five piglets in group 1 were viremic on DPI 35. On DPI 42, viremia was not detected in any animal in group 2, 3, 4, or 5, but two of the piglets in group 1 were viremic. On DPI 49, viremia was detected in group 1 only, in which one of the piglets was viremic and two pigs showed positive staining in the lung samples by immunohistochemistry (see Table 4 below). However, virus was recovered from all living piglets in group 6 at 35, 42, and 49 DPI (Table 2).

**Detection of viral RNA.** To further evaluate the duration of viral RNA of the JXA1-R vaccine strain, serum samples from 0, 3,
**TABLE 4** Histological lesions in different organs of immunized piglets after HP-PRRSV JXA1-F5 challenge

| Group | Lung   | Tonsil | Lymph node | Brain | Heart | Kidney | Spleen | Intestine | Stomach |
|-------|--------|--------|------------|-------|-------|--------|--------|-----------|---------|
| 1     | ++     |        | +++        | 0     | 0     | 0      | 0      | 0         | 0       |
| 2     | 0      | 0      | 0          | 0     | 0     | 0      | 0      | 0         | 0       |
| 3     | 0      | 0      | 0          | 0     | 0     | 0      | 0      | 0         | 0       |
| 4     | 0      | 0      | 0          | 0     | 0     | 0      | 0      | 0         | 0       |
| 5     | 0      | 0      | 0          | 0     | 0     | 0      | 0      | 0         | 0       |
| 6     | 5      | 5      | 5          | 5     | 5     | 5      | 5      | 5         | 5       |
| 7     | 0      | 0      | 0          | 0     | 0     | 0      | 0      | 0         | 0       |

**a** Histological lesions were scored with plus signs. The number of plus signs represents the severity of the lesions.

5, 7, 14, 21, 28, 35, 42, and 49 DPI were evaluated using real-time RT-PCR. The RNA copy numbers in groups 1 to 5 reached their highest levels on DPI 7 and then gradually declined in all groups. On DPI 42, the RNA copy numbers in groups 1 to 5 were not detectable by real-time RT-PCR (Fig. 2A). This was taken as indicative of JXA1-R strain replication levels in the piglets. To further assay quantitative levels of challenge virus replication in the various immunization groups, serum samples from 7, 14, and 21 days postchallenge were analyzed for viral RNA by real-time RT-PCR (Fig. 2B). At 7 days postchallenge, no viral RNA was detected in group 5 or 6, and the RNA copy numbers were significantly lower in groups 2 and 4 than in groups 1 and 6 (Student’s t test, \( P < 0.01 \)). At 14 days postchallenge, no viral RNA was detected in group 2 or 4, and the RNA copy numbers were significantly lower in group 1 than in group 6 (Student’s t test, \( P < 0.01 \)) (Fig. 2B). These results indicated that the viral replication levels of the immunized groups (doses of \( 10^{4.0}/ml \)) were significantly different from those of the challenge control group. Thus, the level of challenge virus RNA in the immunization groups was significantly reduced by the vaccine.

**Clinical signs after immunization and challenge.** General observations and abnormal signs postimmunization and postchallenge were recorded. No piglet immunized with JXA1-R vaccine developed the typical clinical signs of PRRS. After the challenge, the piglets in group 6 exhibited clinical signs typical of PRRS, such as persistently high fever (40.5°C to 42°C) (Fig. 3), depression, anorexia, cough, asthma, lameness, and shivering. Four of the five piglets in group 6 died between days 7 and 14 postchallenge (Table 3). In group 1, only soft coughing and transitory high fever were observed and in only two piglets; both piglets eventually recovered (Fig. 3). None of the piglets in groups 2, 3, 4, 5, and 7 showed high fever or clinical signs of PRRS at any point during the experiment (Table 3).

To assess the gross clinical effects of JXA1-R vaccination and HP-PRRSV challenge on piglets, all animals were weighed on DPI 0, 28, and 49. The average daily weight gain (ADG) in each group was evaluated between 0 to 28 and 28 to 49 DPI (Fig. 4). There was no significant difference in the weight of different treatment groups during days 0 to 28. However, after the challenge, piglets in group 1 gained weight faster than piglets in group 6 (Student’s t test, \( P < 0.05 \)), and piglets in the other groups gained weight even faster than piglets in group 1 (Student’s t test, \( P < 0.01 \)) (Fig. 4).

**Pathological characterization.** To further assess the efficacy of JXA1-R attenuated live vaccine, pathological changes in samples from each group were examined. Typical lesions were found in all piglets in group 6 at necropsy, as were hyperplasia in the lungs, lung hemorrhagic spots, lung edema, blood spots in the kidneys, enlarged lymph nodes with hemorrhagic spots, spleen infarction, and hyperemia of the meninges. In group 1, mild lesions were mainly localized to the lungs, tonsils, and lymph nodes in two of five piglets. No obvious pathological changes were observed in the other immunization groups (Table 4). In addition, histopathological examination was used to observe microscopic lesions. In the challenge control group, animals presented with disease manifestations, including nonsuppurative encephalitis, massive lymphomononuclear infiltration resulting in perivascular cuffing in the brain, and severe inflammation of the lungs, tonsils, lymph nodes, and spleen tissues characterized by infiltrating lymphocytes, macrophages, and necrosis. However, there was mild interstitial pneumonitis in the lungs of two of the piglets in group 1 and slight lymphomononuclear infiltration in lymph nodes and tonsils (Fig. 5). In groups 2 to 5, such histopathological changes were not evident.

**DISCUSSION**

In June 2006, a highly pathogenic PRRSV (HP-PRRSV) called JXA1, which has a unique 30-amino-acid deletion within its Nsp2 coding region, was isolated from diseased piglets in China, where it brought significant economic loss (8). Since then, this PRRSV has become the major epidemic viral strain in China. HP-PRRSV has 89.4% to 97.2% nucleotide identity with North American (NA) PRRSV strains VR-2332, BI-4, CH-1a, HB-1(sh)/2002, and HB-2(sh)/2002. HP-PRRSVs share 98.2% to 100% nucleotide identity overall. These results indicate significant genetic diversity across the HP-PRRSV and the NA-type PRRSV strains. Studies have shown that vaccines protect pigs against homologous or very closely related strains of PRRSV only (23–25). This means that vaccines derived from the NA-type PRRSVs cannot provide completely effective protection against HP-PRRSV. After the outbreak of HP-PRRSV, the HUN4 and TJM vaccine strains provided effective protection in young pigs against the virulent parental HP-PRRSV strain (15, 26). Here, we described a newly developed HP-PRRSV vaccine candidate, JXA1-R, which is efficacious in the prevention of clinical infection caused by the parental JXA1 virus. Whether the JXA1-R can provide cross-protective immunization or be used in a multivalent vaccine remains an interesting question that requires further investigation.

In this report, JXA1-R, a genetically stable attenuated viral strain, was obtained by serially passaging the HP-PRRSV JXA1 viral strain through Marc-145 cells. All nucleotide changes and amino acid mutations relative to JXA1 were observed before the 80th passage. No nucleotide changes or amino acid mutations were observed between the 80th and 100th passages. This indi-
cated that the 80th JXA1 had adapted to the Marc-145 cells and was stably passaged through them. Viruses from the 80th passage were used as seed viruses, and those of the 82nd passage were used for attenuated vaccine development and production.

Our findings indicate that the JXA1-R vaccine can successfully protect piglets against a challenge with HP-PRRSV. First, all piglets in groups 2 to 5 were protected, and all of these animals survived without any major clinical signs at any point in the experimental period. In group 1, two of five piglets exhibited mild coughing and transitory high fever; each piglet eventually recovered. These data suggested that a low dose ($10^{3.0}$ TCID$_{50}$/ml per piglet) of JXA1-R vaccine provided incomplete protection from HP-PRRSV challenge. However, doses of $\geq 10^{4.0}$ TCID$_{50}$/ml per piglet provided full protection against HP-PRRSV. Even high doses of JXA1-R vaccine were found to be safe and effective in these piglets. Second, in this study, the time between vaccination and challenge was 4 weeks. The humoral immune response was assessed by ELISA. All vaccinated piglets had a perceivable humoral immune response to vaccination after day 7. This response promptly increased, almost reaching the maximum S/P ratio value at 28 days. Note also that the neutralizing antibodies generated in some piglets were present only at low levels, and no neutralizing antibodies were detected in certain piglets from groups 1 to 5 (data not shown). These piglets were still effectively protected, yet this suggested that the neutralizing antibodies were not essential for protective immunity. Some researchers reported that the levels of neutralizing antibodies were not always correlated with protection and that animals lacking neutralizing antibodies were nonetheless resistant to reinfection (27, 28). Third, detection of viremia showed that the duration of viremia was about 42 days. HP-PRRSV JXA1 challenge took place 28 days postimmunization. Results indicated that challenge virus replication levels of the immunized groups (doses of $10^{4.0}$/ml) were significantly different from those of the challenge control group. The level of viremia in the immunized groups (doses of $10^{5.0}$/ml) was dramatically reduced during the study (Fig. 2B). Here, JXA1-R vaccination was clearly shown to provide effective protection against challenge with the same virus. The protective response was observed in immunized piglets during the experiment, although the mechanisms of immune protection are still not fully understood. In addition, other experimental studies showed that preventive vaccination with attenuated PRRSV or inoculation with virulent field virus provided a high level of protection against challenge with the same or nearly the same virus (29, 30). Altogether, these results suggest that JXA1-R vaccine is effective against HP-PRRSV infection, but the immunization doses should be $\geq 10^{4.0}$ TCID$_{50}$/ml per piglet. It should be noted when drawing comparisons that the amount of time between vaccination and challenge was 4 weeks in this study versus 3 weeks in other studies (26, 31).

In other studies, an intermediate JXA1 passage (JXA1-F49) was used to inoculate piglets at a dose of $10^{6.5}$ TCID$_{50}$/ml. Two of the five piglets developed clinical symptoms, including rubefaction, cough, and anorexia. Pathological changes included light hypernormal spleen and brain (d and e) are seen. In group 6, massive lymphomononuclear infiltration and hemorrhage in lymph (B), massive lymphomononuclear infiltration and lymphatic follicle scalloping in tonsil (C), massive hemorrhage in spleen (D), and massive lymphomononuclear infiltration surrounding perivascular cuffing (E) are seen.
plasia in the lungs and hemorrhagic spots in the lymph nodes (data not shown). These results indicated that JXA1-F49 still induced pathology and disease but to a lesser extent, suggesting that passaging can further attenuate the virus. However, too many passages might lead to attenuated viruses that cannot provide protection. In one study, high-number JXA1 passages (JXA1-F100, JXA1-F110) were used to inoculate five piglets at a dose of 10^3 TCID_{50}/ml. The animals were then challenged with HP-PRRSV at 28 DPI. No typical clinical signs were seen in the two groups, but light hyperplasia of the lungs was observed in two of five piglets in the JXA1-F110 group (data not shown). This suggested that the JXA1 strain serially passaged in Marc-145 cells may have attenuated the virulence of HP-PRRSV and that attenuated JXA1 strains may have provided protection against HP-PRRSV infection. However, after >100 passages, the efficacy of the JXA1 attenuated strain vaccine gradually decreased.

In our study, JXA1-R (82nd passage) differed from parental JXA1 by 108 nucleotides. No nucleotide or amino acid changes were observed between passages 80 and 100, and almost all of these mutations were scattered throughout the PRRSV genome. Only the coding regions for NSP1α, NSP4 to 6, NSP8, and NSP12 and ORF6 and ORF7 did not contain any mutations (Table 1). These mutations were gradually introduced during the JXA1 viral strain passages through the Marc-145 cells, which showed that the JXA1-R viral strain had fully adapted to the Marc-145 cells and become genetically stable.

In addition, the previous studies suggested that the amino acid substitutions identified are located in functional regions of the PRRSV genome, which are hypothesized to affect the viral replication, signal transduction, protein transport, and antibody neutralization processes associated with PRRSV (32–38). Many of these mutations are not necessarily related to in vivo virulence but rather to adaptation of the viral strain to the culture host cell (32–38). We surmise that the amino acid mutations in the non-structural and structural protein regions of JXA1-R may play key roles in the attenuation of HP-PRRSV in this study.

In conclusion, JXA1-R, a new vaccine against HP-PRRSV, was sufficiently attenuated and capable of providing effective protection against a lethal wild-type HP-PRRSV. However, the detailed molecular basis of protection induced by the JXA1-R vaccine against HP-PRRSV infection requires further study.

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