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ORIGINAL ARTICLE

Genetic and immunogenicity analysis of porcine circovirus type 2 strains isolated in central China

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Abstract

Porcine circovirus type 2 (PCV2) is an economically important pathogen in domestic pigs and wild boars all around the world. To understand the molecular epidemiology of PCV2 strains circulating in central China and to provide a potential vaccine candidate strain, we analyzed the genetic variations of 46 PCV2 isolates circulating from 2009 to 2016 in Henan Province (24 detected in the field from 2009-2013 and 22 from 2013-2016) and evaluated the efficacy of an isolate as a vaccine candidate strain in a mouse model. We found that PCV2b was the predominant genotype and PCV2b-1C was the main subtype. The PCV2 isolate DF-1, which had a virus titer of $10^{6.5}$ $TCID_{50}$/mL and a stable genome, was selected and used to immunize Kunming mice. Enzyme-linked immunosorbent assay (ELISA), immunoperoxidase monolayer assay (IPMA), and virus neutralization test (VNT) results indicated that the DF-1 vaccine candidate strain could elicit a level of specific antibodies and neutralizing antibodies similar to those induced by a commercial vaccine. Polymerase chain reaction (PCR) detection of virus in vaccinated mice after challenge revealed that DF-1 vaccination was effective in clearing the virus in different tissues. Hence, the PCV2 isolate DF-1, a circulating subtype of PCV2b-1C, might be used as a potential vaccine candidate strain.

Introduction

Porcine circovirus type 2 (PCV2) infections are associated with a range of syndromes and diseases, collectively described as porcine circovirus-associated diseases (PCVADs). Post-weaning multisystemic wasting syndrome (PMWS) is the most important PCVAD and is characterized by weight loss, lymphadenectasis, dyspnea, tachypnea, anemia, diarrhea, and jaundice [11, 23, 31]. PCV2, a member of the family *Circoviridae*, is the smallest non-enveloped animal virus with a single-stranded circular DNA, and it has been recognized as one of the most economically threatening pathogens restraining the development of the global swine industry since it was first described in 1991 [20].

The genome of PCV2 is 1,766–1,768 nucleotides in length, containing four major open reading frames (ORFs) [18]. ORF1 encodes the Rep protein, which is associated with viral replication [19], ORF2 encodes the Cap protein, which is the main target for vaccine development [29, 35], and ORF3 encodes a protein that is involved in apoptosis and pathogenesis [14]. A recently discovered viral protein, ORF4 (nt 386 to 565), encodes an apoptosis-suppressing protein that interacts weakly with the Rep protein and suppresses caspase activity during PCV2 infection [7].

Based on ORF2 genes, PCV2 viruses are classified into five genotypes, designated a, b, c, d and e [2, 22]. In recent years, field isolates of PCV2 have mainly been of genotypes a, b and d, and studies on PCV2 genomes have focused on these three genotypes, while genotypes c and e have a lower prevalence. PCV2 experienced two genotype shifts since its discovery. The impact of PCVAD circulating in different countries and

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regions was accompanied in the mid-2000s by a shift in the genotype of the predominant epidemic strains from PCV2a to PCV2b [6]. PCV2a and PCV2b show high similarity at both the genetic and the amino acid level, but PCV2b has been shown to be more pathogenic and antigenic than PCV2a [16, 24]. A second shift resulted in an increase in the prevalence of genotype PCV2d in the United States, Europe, China, Korea and South America. The PCV2c genotype has only been found in Denmark and Brazil [5]. Since the first recognition of PCV2 in 2001, there have been several investigations of the distribution and prevalence of PCV2 in different areas of China [10, 21, 28]. The prevalence trend of PCV2 is in line with that of other areas, with PCV2a, PCV2b, PCV2d being the predominant genotypes in China. PCV2d is a newly emerging genotype that has been circulating in China since 2010, but the PCV2b genotype is still the most common variant [34]. It is worth noting that three strains have been found to be potential recombinants of PCV2b and PCV2c with high homology to PCV2c, which suggests that they had been imported into China [17]. The swine population in central China has suffered severely with PCV2 infections, and multiple strains sometimes infect the same pig [9, 21, 32].

Currently, vaccination is the main tool for controlling PCVAD in swine populations [13]. Commercial vaccines include subunit vaccines, live-attenuated PCV1-2a or 2b chimera vaccines, and conventional inactivated PCV2 vaccines [8]. In China, there are at least six vaccines that are commercially available for veterinary use. Five of them are inactivated vaccines (one PCV2a vaccine, one PCV2d vaccine, and three PCV2b vaccines), and these inactivated vaccines, especially the PCV2b vaccines, still dominate the market [33]. Although PCV2 vaccines are able to induce an immune response that protects against both predominant PCV2 genotypes, PCV2 vaccines based on genotype PCV2b are more effective than those based on PCV2a [3, 4, 25, 26]. Moreover, it has been demonstrated that genetic differences among different PCV2 isolates strains could affect virulence and antigenicity directly, affecting vaccination efficacy, pathogenesis and disease diagnosis [15]. To understand the molecular epidemiology of PCV2 in central China and provide a potential vaccine candidate based on the main circulating strain, we analyzed the sequences of 46 Chinese isolates of PCV2 collected from 2009 to 2016 (including 24 isolates and 22 reference sequences from the GenBank database) and evaluated the efficacy of a potential vaccine candidate in a Kunming mouse model.

Materials and methods

Field samples

A total of 54 tissue samples were collected from pigs at 5 to 22 weeks of age from 39 farms with suspected PMWS in Henan province between 2009 and 2013. Of these samples, 25 were from spleens, 17 were from lymph nodes, 10 were from lungs, and two were from kidneys. The tissue samples were completely ground under aseptic conditions, and total DNA was extracted using a QIAamp® DNA Mini Kit (QIAGEN, Germany).

Virus isolation

A PCV2 virus isolate was grown in pig kidney-15 (PK-15) cells grown in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone), penicillin and streptomycin (Pen-Strep) (InvivoGen, France), and L-glutamine at 37 °C in a 5% CO_2 atmosphere. Initially, 200 µL of each tissue homogenate was allowed to adsorb to monolayer cells in 12-well plates for 1 h and then poured into DMEM with 2% FBS, L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, and Pen-Strep. Cells were harvested after 5 days of incubation. Subsequent passages were performed by applying 200 µL of cell culture onto confluent monolayers in 12-well plates as described above. Viral replication of each generation was verified by PCR and immunoperoxidase monolayer assay (IPMA).

Immunoperoxidase monolayer assay (IPMA)

IPMA was performed for virus isolation and titration. Briefly, isolated viruses were grown in PK-15 monolayers in 96-well plates for 24 h in DMEM containing 2% FBS, and the cells were the fixed in cold paraformaldehyde containing 0.5% Triton X-100 and 1.0% H_2O_2 for 15 min. After blocking with 5% skimmed milk at 37 °C for 1 h, polyclonal rabbit anti-PCV2 antibodies (Veterinary Medical Research & Development (VMRD), USA) was diluted 1:800 and incubated with the infected cells at 37 °C for 1 h. Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG at a dilution of 1:3000 was then incubated with the cells as above and used as secondary antibodies. A chromogenic reaction was performed using an AEC kit, and the plates were observed under a light microscope. After each step, the plates were washed 3 times with PBS containing 0.05% Tween 20 (PBST). The Reed-Muench method was used to calculate the 50% tissue culture infective dose ($TCID_{50}$/mL).

Sequencing and phylogenetic analysis

ORF2 genes were amplified using the following primers: forward, 5'-CGG ATA TTG TAG TCC TGG TCG-3'; reverse, 5'-ACT GTC AAG GCT ACC ACA GTC A-3'. Complete genomic DNA was amplified using the following primers: forward, 5'-TAT CCG CGG GCT GGC TGA ACT TTT GAA-3'; reverse, 5'-GTG CCG CGG AAA TTT CTG

ACA AAC GTT-3'. The amplified PCR products were purified using a Gel Extraction Kit (OMEGA Bio-Tec., China) and ligated into the pMD19-T vector (Takara, Japan). After transformation of competent *E. coli* DH5α cells, a single colony was identified by PCR and restriction enzyme digestion and confirmed by sequencing. A total of 24 complete genome sequences were determined and submitted to GenBank after editing with BioEdit software (Table 1). BLAST analysis (NCBI) was performed to identify gene homologs among the complete genomes. The sequences were aligned by the Clustal W algorithm using the MegAlign program in the DNAStar software package (DNASTAR, USA), and phylogenetic trees were constructed by the neighbor-joining analysis with 1000 bootstrap replicates and the maximum composite likelihood method, using MEGA 5.1 software. The phylogenetic datasets for sequence analysis included 22 other sequences from isolates collected from Henan province during 2009-2016, eight reference sequences (PCV2a, PCV2b-1A, PCV2b-1A1B, PCV2b-1C, PCV2d, PCV2c, PCV1), three vaccine strains (LG, SH, DBN-SX07) from China, and two reference PCV2a strains of AF027217 (Canada) and AF381175 (China) (Table 2). Amino acid sequences of the ORF2-encoded Cap protein from these isolates were also analyzed and aligned using DNAStar software.

Selection of a potential candidate vaccine strain

To select a potential vaccine strain, 13 PCV2 isolates obtained in this study were passaged in PK-15 cells. Virus titers were determined by IPMA after 5, 10, 15, 20 and 25 passages and expressed as $\log_{10}TCID_{50}$/mL. Whole-genome sequencing was performed after 4, 15, and 25 passages to examine the genetic stability of the isolates. A potential vaccine candidate strain with a higher virus titer in IPMA and a more stable genome was selected, and its efficacy as a vaccine was tested in a mouse model. The virus was inactivated with 0.3% formaldehyde for 36 h and emulsified with 94% (v/v) mineral oil, 6% (v/v) Span-80, and 2% (g/v) aluminum stearate for vaccine preparation.

Animal experiment

Sixty 4-week-old female Kunming mice were purchased from the Experimental Animal Breeding Center of Zhengzhou University, and randomly divided into three groups

Table 1 PCV2 isolates collected in Henan province between 2009 and 2013

GenBank accession number	Region	Isolation year	Genome size (nt)	Genotype	ORF2 length (nt)	Isolate name
HQ693092	Nanyang	2009	1768	2b-1A	702	NY-1
HQ693093	Zhengzhou	2010	1767	2b-1C	705	Zhengzhou
HQ650833	Wuzhi	2010	1767	2b-1C	705	WZ-1
JF899334	Henan	2011	1767	2b-1C	705	HN-2
JF928002	Xinzheng	2011	1767	2b-1C	705	XN-1
JF928003	Pingdingshan	2011	1768	2a	702	PDS-1
JF928004	Linying	2011	1767	2b-1C	705	LY-2
JF928005	Luoyang	2011	1767	2b-1A	702	LN-1
JF928006	Nanyang	2011	1767	2b-1C	705	NY-2
JN119255	Dengfeng	2011	1767	2b-1C	705	DF-1
JN119256	Wenxian	2011	1767	2b-1A	702	WX-1
JN119257	Zhongmou	2011	1767	2b-1C	705	ZM-4
JN615187	Jiyuan	2011	1767	2b-1C	705	JY-2
KC684978	Xingyang	2012	1767	2b-1A	702	XY
KC753768	Dengfeng	2012	1767	2b-1C	705	DF
KC753769	Dengfeng	2012	1767	2b-1C	705	DF-2
KC753770	Jiyuan	2012	1767	2b-1C	705	JY
KC753771	Jiyuan	2012	1767	2b-1C	705	JY-1
KC753772	Zhengzhou	2012	1767	2b-1C	705	ZZ-1
KC821781	Jiaozuo	2013	1767	2b-1C	705	JZS-1
KC821782	Jiaozuo	2013	1767	2b-1C	705	JZS-2
KC821783	Jiaozuo	2013	1767	2b-1C	705	JZS-7
KC821784	Jiaozuo	2013	1767	2b-1C	705	JZB-2
KC821785	Jiaozuo	2013	1767	2b-1C	705	JZB-7

Table 2 Reference sequences used in the bioinformatics analysis

GenBank accession number	Region	Isolation year	Genome size (nt)	Genotype	ORF2 length (nt)	Isolate name
AY686763	Shanghai	Unknown	1767	2b-1C	705	SH
HM038034	China	2008	1768	2a	702	LG
HM641752	China	2007	1767	2b-1A	702	DBN-SX07-2
HM038030	China	2008	1766	2b-1C	705	AH
AY181946	Tianjin	Unknown	1767	2b-1C	705	TJ
DQ206444	Rizhao	Unknown	1767	2b-1A1B	702	JZ
AF055394	France	Unknown	1767	2b-1A	702	-
DQ141322	Shandong	Unknown	1767	2b-1A	702	SD1
AF055392	Canada	Unknown	1768	2a	702	-
EU148503	Denmark	Unknown	1767	2c	705	DK1980PMWSfree
AY184287	Unknown	Unknown	1759	PCV1	702	-
AF381175	China	Unknown	1768	2a	702	BF
AF027217	Canada	Unknown	1768	2a	702	pmws PCV
GU938302	Nanyang	2009	1767	2b-1C	705	NY0912
JX534236	Henan	2012	1767	2b-1A	702	HALY12
JX534237	Henan	2012	1767	2b-1A	702	HALY12
GU450328	Henan	2009	1767	2b-1A	702	HEN0901
KM035761	Henan	2014	1767	2b-1A	702	HN-2
KM035762	Henan	2014	1767	2a	702	HN-6
KM067384	Henan	2014	1767	2b-1A	702	HN-12
KM067385	Henan	2014	1767	2a	702	HN-13
KF926650	Xinzheng	2014	1767	2b-1A	702	XZBK
KU960929	Hebi	2016	1767	2b-1C	705	HB
KU960930	Zhouko	2016	1767	2b-1C	705	HY-1
KU960931	Zhouko	2016	1767	2b-1A	702	HY-2
KU960932	Jiaozuo	2016	1767	2b-1C	705	JZ-1
KU960933	Jiaozuo	2016	1767	2b-1C	705	JZ-2
KU960934	Jiaozuo	2016	1767	2b-1A	702	JZ-8
KU960935	Kaifeng	2016	1767	2b-1C	705	KF-1
KU960936	Kaifeng	2016	1767	2b-1C	705	KF-2
KU960937	Luoyang	2016	1767	2b-1C	705	LY
KU960938	Zhumadian	2016	1767	2b-1A	702	PY
KU960939	Zhumadian	2016	1767	2b-1C	705	RN
KU960940	Shangqiu	2016	1767	2b-1C	705	SQ
KU960941	Luoyang	2016	1767	2b-1C	705	YY

with 20 in each group. Mice in group DF-1 were injected subcutaneously with 200 μL of the inactivated DF-1 strain (6.5 $\log_{10}$ $TCID_{50}$/mL). Mice in group CV were injected with 200 μL of a commercial inactivated vaccine (Ingelvac CircoFLEX®, Germany). Mice in the control group received 200 μL of DMEM. A booster immunization was given with the same amount of immunogen at 28 days after the primary immunization. 28 days after the booster immunization, all groups were challenged intramuscularly with 0.5 mL of PCV2 strain DF-1 (5.0 $\log_{10}$ $TCID_{50}$/mL). Serum samples were collected weekly after the primary immunization until the twelfth week, and antibody titers were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Wuhan Keqian Animal Biology Product Co., Ltd, China), IPMA, and virus neutralization test (VNT). Antibody titers were expressed as the OD S/P ratio, the highest dilution of IPMA, and $\log_2$NA, respectively. Cytokines IL-10, IL-18, IFN-γ, TNF-α, and GM-CSF were measured using a commercial cytokine ELISA kit (USCN Life Science, China) to evaluate the level of cellular immunity. PCR was used to detect the presence of PCV2 in the lungs, spleens, hearts, livers and kidneys of the challenged mice.

Virus neutralization test (VNT)

All serum samples were assessed for their ability to neutralize the PCV2 strain DF-1 using a neutralization assay. Briefly, 50 μL of serum was pre-treated at 56 °C for 30 min, diluted in a two-fold series from 1:2 to 1:1024, mixed with an equal volume of virus (400 $TCID_{50}$), and incubated at 37 °C for 1 h. The serum-virus complex was transferred onto confluent PK-15 cells in multi-well plates and incubated at 37 °C for 72 h. As no visible cytopathic effect was observed, IPMA was performed to detect the presence of virus. The virus neutralization titer was expressed as the highest dilution (log_2NA) at which no more than 80% reduction of virus replication was detected compared to the virus control.

Statistical analysis

One-way analysis of variance (ANOVA) was performed using GraphPad software. Data are shown as the mean ± SEM, and the level of significance for all statistical tests was set at 0.05 ($p < 0.05$).

Results

Phylogenetic analysis

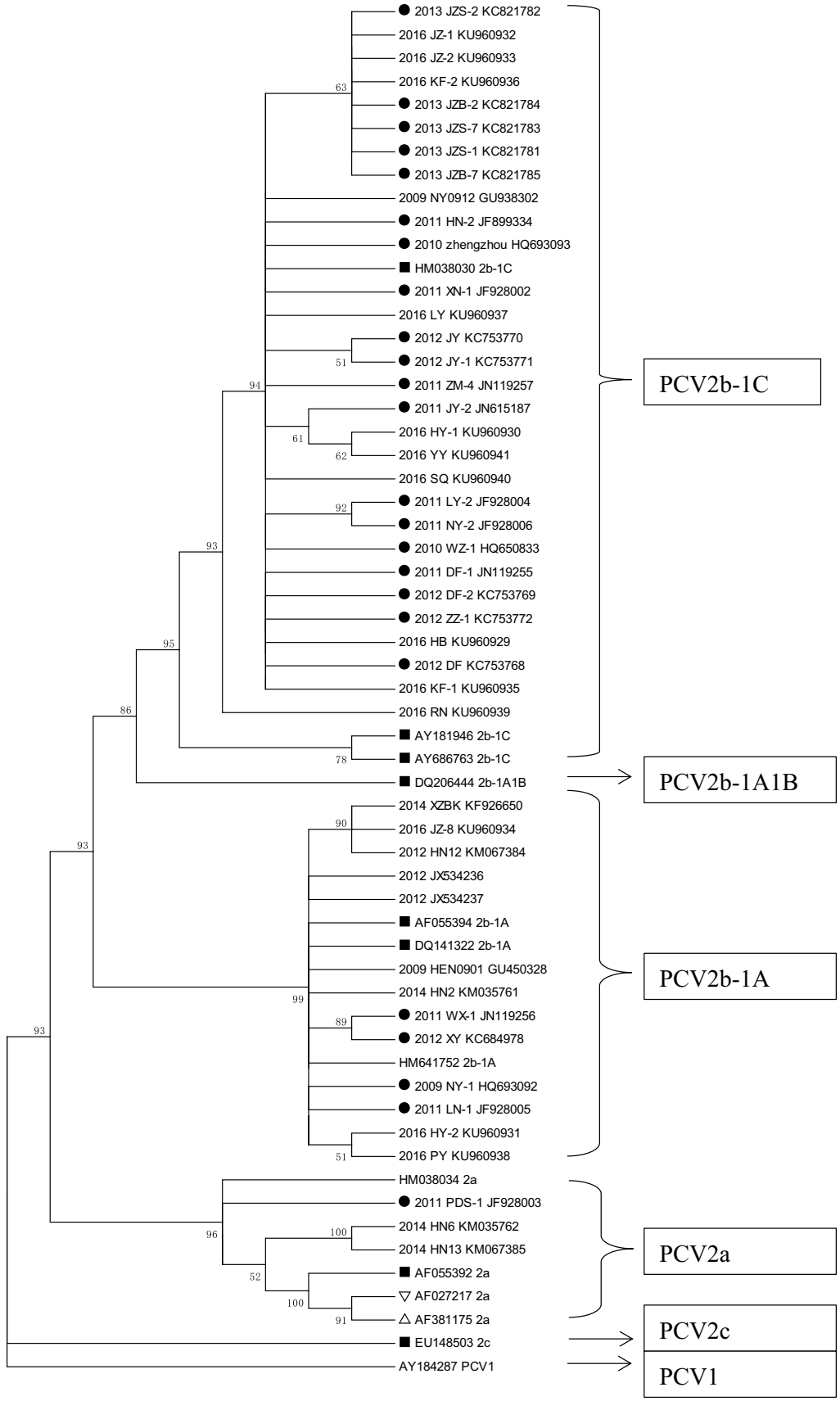
Twenty-four PCV2 isolates were identified in a survey of 39 farms. The genomes of these isolates were 1767 or 1768 bp in length. The pairwise sequence identity values for all 46 Henan strains (including 24 isolates and 22 reference sequences from the GenBank database) ranged between 94.2% and 99.9%. Compared with the representative PCV2a strains AF027217 (Canada) and AF381175 (China), the nucleotide sequence identity of the 46 isolates ranged from 94.9% to 96.8% and 95.0% to 97.5%, respectively. Phylogenetic analysis based on ORF2 genes indicated that two distinct genetic groups, PCV2a and PCV2b, were circulating in Henan Province. Twenty-three out of 24 isolates from 2009 to 2013 belonged to the PCV2b genotype, indicating that PCV2b was the main genotype in central China 4-8 years ago. Twenty out of 22 isolates from 2013 to 2016 were of the PCV2b genotype, indicating that PCV2b continued to be the main genotype circulating in this area (Fig. 1). The genetic distances among the isolates ranged from 0.001 to 0.053. Moreover, further analysis showed that 32 of these PCV2b isolates belonged to subtype PCV2b-1C, and 13 strains belonged to PCV2b-1A. Comparison of complete genomic sequences revealed 95.7-99.9% sequence identity among PCV2b-1C sequences. The PCV2b-1C and PCV2b-1A sequences were 96.6-98.6% identical, and the sequences of the newly analyzed PCV2b-1A isolates were 98.6-99.5% identical to each other.

To investigate the possible effect of individual amino acid changes on virulence, the amino acid sequences of the 46 PCV2 isolates from central China were aligned and compared with those of the vaccine strains SH, LG and DBN-SX07, belonging to subtype 2b-1C, 2a and 2b-1A, respectively. Alignment of the predicted amino acid sequences of the ORF2 protein revealed that the 46 isolates shared 84.2-99.9% identity. There were six major regions of variation within these ORF2 sequences (amino acids 53-68, 89-90, 131-136, 167-169, 185-191, and 206-215) (Fig. 2). Specifically, the three isolates of PCV2a carried substitutions at position 21 (L to Q), 51 (C to R), 72 (M to L), 77 (N to D), 139 (G to D), 185 (L to M), 200 (I to T), and 206 (T to K). The amino acids at positions 131-135 in the three PCV2a isolates were completely different from each other, but the corresponding region was relatively conserved among the PCV2b isolates. In addition, seven out of 13 PCV2b-1A isolates had amino acid variations at positions 30 (V to L), 59 (R to K), 63 (K to R), and 190 (A to T). All the PCV2b-1C isolates carried amino acid changes at positions 8 (Y to F), 34 (L to H), 59 (A to K), 68 (A to N), 151(P to T), 169 (R to G/S), and 206 (K to I).

Evaluation of the efficacy of a potential candidate vaccine strain

Thirteen strains were picked as stably cultured and passaged. Among the 24 field isolates, PCV2 DF-1 showed the best growth kinetics, reached $10^{6.5}$ $TCID_{50}$/mL in the fifteenth passage, and was stably maintained for 25 generations or longer (Fig. 3). Whole-genome sequencing of PCV2 strain DF-1 showed that the nucleotide sequence remained unchanged at each passage, indicating that the isolate DF-1 had a stable genome.

An ELISA for measuring the IgG level showed that PCV2-specific antibodies appeared at 21 days after the primary inoculation (dpi) in groups DF-1 and CV and increased significantly until 35 dpi. Antibodies in group DF-1 remained at high levels from 35 dpi to 84 dpi. It is noteworthy that the antibody level in group CV decreased from 35 dpi and remained at a lower level than DF-1 until PCV2 challenge (Fig. 4a). Serum antibodies were not detected 14 days after the primary immunization because the antibody level was too low to be detected. There was no difference in the IPMA titers of serum antibodies between group DF-1 and group CV before the booster immunization. IPMA titers of serum antibodies in group DF-1 remained higher than those in group CV from 7 days after the booster immunization until challenge, which was consistent with the ELISA results (Fig. 4b). PCV2 neutralizing antibodies in group DF-1 also remained higher than those of group CV during the entire experiment (Fig. 4c). This might due to the fact that the immunogen of group DF-1 was same to that used for


2013 JZS-2 KC821782
2016 JZ-1 KU960932
2016 JZ-2 KU960933
2016 KF-2 KU960936
2013 JZB-2 KC821784
2013 JZS-7 KC821783
2013 JZS-1 KC821781
2013 JZB-7 KC821785
2009 NY0912 GU938302
2011 HN-2 JF899334
2010 zhengzhou HQ693093
HM038030 2b-1C
2011 XN-1 JF928002
2016 LY KU960937
2012 JY KC753770
2012 JY-1 KC753771
2011 ZM-4 JN119257
2011 JY-2 JN615187
2016 HY-1 KU960930
2016 YY KU960941
2016 SQ KU960940
2011 LY-2 JF928004
2011 NY-2 JF928006
2010 WZ-1 HQ650833
2011 DF-1 JN119255
2012 DF-2 KC753769
2012 ZZ-1 KC753772
2016 HB KU960929
2012 DF KC753768
2016 KF-1 KU960935
2016 RN KU960939
AY181946 2b-1C
AY686763 2b-1C
DQ206444 2b-1A1B
2014 XZBK KF926650
2016 JZ-8 KU960934
2012 HN12 KM067384
2012 JX534236
2012 JX534237
AF055394 2b-1A
DQ141322 2b-1A
2009 HEN0901 GU450328
2014 HN2 KM035761
2011 WX-1 JN119256
2012 XY KC684978
HM641752 2b-1A
2009 NY-1 HQ693092
2011 LN-1 JF928005
2016 HY-2 KU960931
2016 PY KU960938
HM038034 2a
2011 PDS-1 JF928003
2014 HN6 KM035762
2014 HN13 KM067385
AF055392 2a
AF027217 2a
AF381175 2a
EU148503 2c
AY184287 PCV1
63
51
94
61
62
92
93
95
86
78
90
93
99
89
93
96
100
52
100
91
PCV2b-1C
PCV2b-1A1B
PCV2b-1A
PCV2a
PCV2c
PCV1

◂**Fig. 1** Phylogenetic analysis based on the ORF2 genes of the PCV2 strains, including the 24 isolates from this study and 35 strains whose sequences were obtained from GenBank. The accession numbers for these strains are indicated, and some branches in the tree are collapsed in the form of black triangles, followed by their corresponding year and accession numbers. The tree was constructed by the neighbor-joining method in MAGE 5.1 software. Bootstrap values from 1,000 replications are shown at each node. Filled circles "●" represent isolates from this study, filled triangles "▲" represent reference vaccine strains, filled squares "■" represent reference genotype strains, unfilled regular triangles "△" represent PCV2a strains of AF381175 (China), and unfilled inverted triangles "▽" represent PCV2a strains of AF027217 (Canada). The other strains with no symbols isolate from 2014 to 2016 in central China

detection of antibodies. Notably, mice in the group receiving DMEM alone produced PCV2-specific antibodies and neutralizing antibodies from 7 days post-challenge, detected by ELISA, IPMA, and VNT, which indicated that the virus could stimulate animals without background immunity to produce antibodies. The induction of IL-10, IL-18, GM-CSF, IFN-γ, and TNF-α in serum samples from these three groups was also investigated. However, no significant differences were found in the levels of these cytokines among the three groups (data not shown).

Four weeks post-challenge, fresh tissues of heart, liver, spleen, lung, and kidney from each mouse were taken aseptically and subjected to PCR amplification. The detection rate of positive animals (4/20) was equal for group DF-1 and group CV. PCV2 remained in lung and kidney in four mice of group DF-1 but maintained in lung, kidney, liver, and spleen in four mice of group CV, indicating that immunization with PCV2 isolate DF-1 was as effective for controlling virus spread *in vivo* as the commercial vaccine. More than half of the mice in the group receiving DMEM alone carried the virus in lungs, and PCV2 could be found in all the examined tissues in this group (Table 3). PCV2 isolate DF-1 thus shows potential as a vaccine strain.

Discussion

PCV2 infection is widespread in central China, causing huge economic losses for the swine industry each year. Commercial subunit PCV2 vaccines and inactivated vaccines based on PCV2a and PCV2b have been widely used since 2009 in China [33]. However, most farms in central China still experience subclinical infections with PCV2. It has been suggested that genetic variation might cause vaccine failure, which has been demonstrated for influenza virus [12], but there is no direct evidence that this occurs with PCV2. It has been suggested that genetic variation of PCV2 may have an impact on virulence that is relevant to vaccination, pathogenesis and diagnosis [30]. Hence, it is necessary to investigate the sequence variability of PCV2 isolates circulating in central China and provide a vaccine candidate strain based on the main circulating strain. In this study, we performed phylogenetic analysis based on the sequences of 46 PCV2 isolates collected from 2009 to 2016 in Henan Province (24 sequences from isolates collected by our group from 2009 to 2013 and 22 sequences from the GenBank database from isolates from 2013 to 2016), and we evaluated the efficacy of one isolate, DF-1, as a potential vaccine candidate strain in a mouse model.

Phylogenetic analysis showed that 87.5% of the new isolates belonged to genotype PCV2b-1C with the SH vaccine strain, and 4.2% of the new isolates were of the same genotype, PCV2a, as the LG vaccine strain. This indicated that PCV2b was predominant in central China from 2009 to 2016, and PCV2b-1C was the main subtype, which is consistent with the results of a previous study of the molecular epidemiology of PCV2 in China from 2009 to 2010 [1]. Comparisons of the complete genomic sequence revealed 95.7-99.9% sequence identity among PCV2b-1C sequences. The nucleotide and amino acid sequence similarity among the isolates from the same region (isolates JZS-1, JZS-2, JZS-7, JZB-2 and JZB-7) or isolates obtained at different times (isolates DF, DF-1 and DF-2) in the same region was low, indicating that multiple strains were circulating in the field. Although cross-protection among different genotypes have been observed, many studies have shown that vaccines based on the genotype of the circulating strain work more efficiently in controlling PCV2 infections [27]. Therefore, we selected a potential vaccine candidate strain based on the circulating PCV2 isolates of genotype PCV2b and tested its efficacy.

After the successful isolation and passages of PCV2 isolates, DF-1 showed the best growth kinetics, and the viral titer reached $10^{6.5}$ $TCID_{50}$/mL. DF-1 shared over 95% nucleotide sequence identity with circulating strains in Henan Province from 2009-2016. In addition, complete genome sequencing of DF-1 up to the twenty-fifth passage showed that its genome was stable with no shifts or mutations in the nucleotide sequence. The high degree of genetic homology and stability makes it a suitable vaccine candidate for field use. Hence, we investigated the efficacy of an inactivated vaccine based on the DF-1 strain in a mouse model. Inactivated DF-1 was subcutaneously injected after emulsification with adjuvant. Using DF-1 as detecting antigen, ELISA and IPMA showed that antibody titers in DF-1-immunized mice remained slightly higher than in those immunized with the commercial vaccine from 7 days after the booster immunization until challenge. PCV2 neutralizing antibodies tested by VNT also showed the same trend, and PCR measurement of the virus load in different tissues indicated that mice vaccinated with DF-1 were as competent in clearing the virus as mice with immunized the commercial vaccine. There is thus a good correlation between *in vitro* neutralization

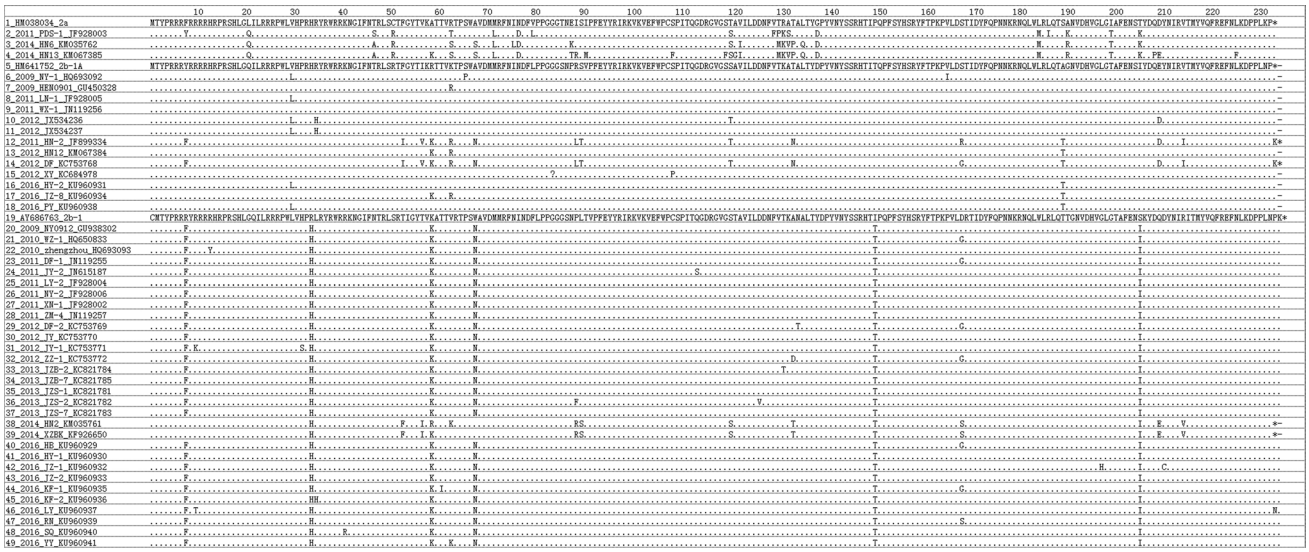


Fig. 2 Alignment of the deduced amino acid sequences of the capsid genes of 46 PCV2 strains isolated in Henan province from 2009 to 2016. Residues that match the consensus sequence are indicated by dots

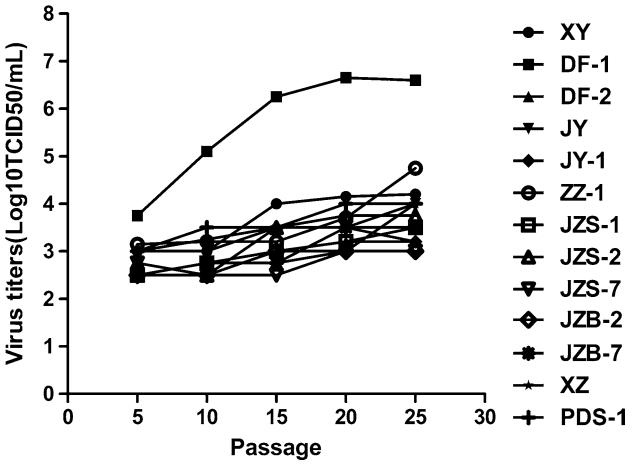


Fig. 3 Growth kinetics of different isolates, Log10 transformed for different isolates of IPMA

and protection against infection with the epidemic PCV2 strain. In view of the fact that the current vaccine based on PCV2a, 2b and 2d provides cross-protection against different genotypes of PCV2 and that PCV2b has been shown to be more pathogenic and antigenic than PCV2a [16, 24], the aim of this study was to find an inactivated candidate vaccine strain in order to combat the current epidemic caused by this virus. Moreover, PCV2 spread in hearts, livers, kidneys, lungs, and spleens of the challenged mice in the group receiving DMEM alone, further confirming that a mouse model is suitable for evaluating PCV2 infection. Subsequent animal experiments on swine will be performed soon. We observed that the levels of cytokines IL-10, IL-18, IFN-γ, TNF-α, and GM-CSF did not differ significantly and showed no meaningful trend. This might be due to the low sensitivity of the ELISA test. The determination of PCV2-specific cytokine-producing splenocytes by ELISPOT assay

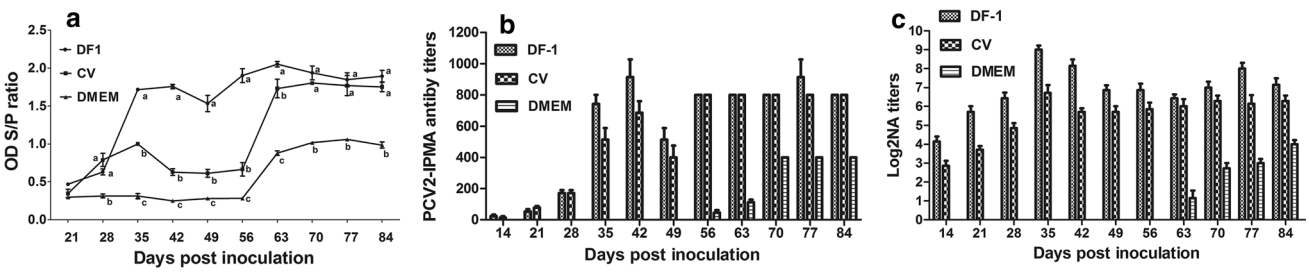


Fig. 4 Detection of PCV2-specific immune responses. OD S/P ratios were determined using a commercial ELISA kit for PCV2-specific IgG (a), IPMA (b), and neutralizing antibodies (NA) (c) for three different groups (vaccinated with experimental inactivated PCV2 DF-1 strain and challenged with strain PCV2 DF-1 56 days after vaccination [group DF-1], vaccinated with commercial inactivated PCV2 vaccine [Ingelvac CircoFLEX®, Germany] and challenged with strain PCV2 DF-1 56 days after vaccination [group CV], or inoculated with DMEM as a control and challenged with strain PCV2 DF-1 56 days after vaccination [group DMEM]). Different letters (a, b, and c) indicate statistically significant differences ($p < 0.05$) among groups

Table 3 Detection of PCV2 at 4 weeks post-challenge

Group	Number of PCV2-positive tissue samples				
	Heat	Liver	Spleen	Lung	Kidney
DF-1	0	0	0	3	1
CV	0	1	1	4	2
DMEM	6	8	8	12	6

or relative quantitation of gene expression by RT-PCR might be more useful for finding differences.

In this study, we analyzed 46 PCV2 isolates circulating in central China from 2009 to 2016 and found that PCV2b was the predominant genotype and PCV2b-1C was the main subtype. Evaluation of the efficacy of an inactivated PCV2 DF-1 isolate with fast growth kinetics and a high virus titer in a mouse model showed that it was effective for eliciting the production of antibodies, including neutralizing antibodies, indicating that it might be used as a vaccine candidate.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest.

Ethics approval The animal experiments were carried out according to the Animal Experiment Committee of Henan Academy of Agricultural Sciences. All animals received humane care in compliance with good animal practice according to the animal ethics procedures and guidelines of China.

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