A recombinant porcine circovirus type 2 expressing the VP1 epitope of the type O foot-and-mouth disease virus is infectious and induce both PCV2 and VP1 epitope antibodies

Liping Huang · Feiyan Zhang · Qinghai Tang · Yanwu Wei · Hongli Wu · Longjun Guo · Yujie Fu · Changming Liu

Abstract Porcine circovirus type 2 (PCV2) is the etiological agent of postweaning multisystemic wasting syndrome, a disease that causes huge economic damage in swine industry. A recombinant PCV2 expressing the neutralizing VP1 epitope (aa 141–160) of the foot-and-mouth disease virus (FMDV) was rescued using an infectious cloning technique. The PCV2 antigen and FMDV-VP1 antigenic epitope of the cloned strain recPCV2-CL-VP1 were confirmed by an immunoperoxidase monolayer assay (IPMA) and a capture enzyme-linked immunosorbent assay (ELISA). The morphological features of the recPCV2-CL-VP1 were not discernibly different from those of its parental strain (PCV2-CL). However, the recombinant virus could be differentiated from its parental virus by PCR and capture ELISA. The recPCV2-CL-VP1 was demonstrated to replicate stably in PK-15 cells through ten passages. An infection experiment using BALB/c mice showed that both recPCV2-CL-VP1 and PCV2-CL could replicate in the mice, cause various pathological changes, and induce a high level of anti-Cap antibodies. The recombinant virus emulsified with Freund's adjuvant was used to immunize BALB/c mice and induced antibodies against the FMDV-VP1 epitope. Hence, the recombinant PCV2 strain, which expressed the neutralizing FMDV-VP1 epitope, provides a valuable platform to develop novel genetic vaccines.

Keywords Porcine circovirus type 2 · Foot-and-mouth disease virus · VP1 · Neutralizing epitope · Recombinant virus

Liping Huang and Feiyan Zhang contributed equally to this study.

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Introduction

Porcine circovirus type 2 (PCV2) is a member of the genus *Circovirus* of the family *Circoviridae* and has been confirmed as the etiologic agent of postweaning multisystemic wasting syndrome (PMWS) (Meehan et al. 1998). Besides PMWS, PCV2 can also cause porcine reproductive disorders, porcine dermatitis, nephropathy syndrome, necrotic lymphadenitis, and porcine respiratory disease, which are collectively termed as porcine circovirus disease (PCVD) (Chae 2005). PCVD has been reported worldwide and causes enormous economic losses to the pork industry (Allan and Ellis 2000; Segalés et al. 2005; Gillespie et al. 2009). PCV2 is an extremely small non-enveloped virus with an icosahedral capsid that measures only 17 nm in diameter containing a circular single-stranded DNA genome of only 1,766–1,769 nucleotides (Allan et al. 1998; Huang et al. 2011a), which encodes two major open

reading frames (ORFs), ORF1 and ORF2. ORF1 encodes two replication-associated proteins, whereas ORF2 encodes the viral capsid (Cap) protein, which is involved in virion assembly and infection, and presents an important antigen for the vaccine development (Nawagitgul et al. 2000; Mankertz et al. 2004).

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals, such as cattle and pigs, caused by the foot-and-mouth disease virus (FMDV) and usually leads to severe economic losses (Grubman and Baxt 2004). FMDV belongs to the *Aphthovirus* genus of the *Picornaviridae* family and contains a capsid composed of 60 copies of four nonidentical virion polypeptide chains: 1A (VP4), 1B (VP2), 1C (VP3), and 1D (VP1) (Doel and Collen 1982). VP1 can induce neutralizing antibodies in experimental and natural hosts through two immunogenic sites on the VP1 protein located at amino acid (aa) residues 141–160 and 200–213 (Kupper et al. 1981; Saiz et al. 2002; DiMarchi et al. 1986; Steward et al. 1991; Zamorano et al. 1994). Bittle et al. (1982) synthesized a peptide comprising aa 140–160 of the type O FMDV-VP1 protein, which can induce the generation of neutralizing antibodies in vaccinated guinea pigs. A multiepitope recombinant vaccine, containing three copies each of residues 141–160 and 200–213 of the FMDV O/China/99 strain VP1 protein coupled with the swine immunoglobulin G heavy-chain constant region against FMDV type O, elicited high titers of anti-FMDV-specific antibodies in swine at 30 days post-vaccination and conferred complete protection (Shao et al. 2011).

A single hemagglutinin (HA) tag, dimer HA tag, trimer HA tag, a Glu-Glu epitope tag from the mouse polyomavirus medium T-antigen (CEEEEYMPME), the KT3 tag from the simian vacuolating virus 40 large T-antigen (KPPTPPPEPET), and the V5 epitope derived from simian virus 5 were inserted, respectively, into the C-terminal of the PCV2 Cap protein and a series of recombinant chimeric viruses with tags were constructed (Beach et al. 2011; Huang et al. 2011b). Those recombinant viruses were infectious and elicited anti-PCV2 neutralizing antibodies and anti-epitope tag antibodies (Beach et al. 2011; Huang et al. 2011b). Therefore, we questioned whether PCV2 could accept the epitope region (aa 141–160) of FMDV-VP1? Until now, related research was not reported.

In the present study, the epitope region (aa 141–160) of FMDV-VP1, for the first time, was inserted into the C-terminal of the PCV2-Cap protein to construct a recombinant PCV2 strain. The FMDV-VP1 epitope was presented on the surface of the PCV2 Cap protein. The recombinant virus could be distinguished with its parental virus by PCR and capture ELISA. Furthermore, the immunogenicity of recombinant virus was confirmed in BALB/c mice, and results showed that the virus could induce anti-PCV2-Cap protein antibodies and anti-FMDV-VP1 antibodies. This study provides a foundation for the development of novel recombinant genetic PCV2 vaccine.

Materials and methods

Viruses, plasmids, and cells

The epitope region (aa 141–160, LTNVRGDLQVLAQKAARPLP) of FMDV-VP1 protein (GenBank accession HM229661) was inserted into the C-terminal of the PCV2-Cap protein. PCV2-CL strain was isolated originally from a pig with clinical PMWS manifestation and its GenBank accession is HM038033. pMD18/PCV2-CL was constructed by cloning the entire genome of PCV2-CL strain into the plasmid pMD18-T (Takara, Dalian, China) and PCV2-CL was rescued as described previously (Guo et al. 2010). PCV-free porcine kidney (PK)-15 cells were grown in minimal essential medium (MEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10 % heat-inactivated fetal bovine serum and 100 g/mL of penicillin and streptomycin at 37 °C. Three millimoles of D-glucosamine (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium for PCV2 propagation. PK-15 cells were used for genomic DNA transfection, viral propagation, and titration.

Antibodies

PCV2-positive and -negative serum, and horseradish peroxidase (HRP)-labeled anti-PCV2-Cap protein monoclonal antibody (HRP-mAb-1D2) were produced according to the methods of Huang et al. (2011c). The anti-FMDV monoclonal antibody (HRP-anti-FMDV mAb) was included in an enzyme-linked immunosorbent assay (ELISA) kit for detection of antibodies against FMDV serotype O in the sera of cattle, sheep, goats, and pigs (Ceditest; Prionics Lelystad B.V., Lelystad, Netherlands).

Construction of the recombinant plasmid

Fusion PCR was performed to construct a recombinant plasmid containing the VP1 epitope sequence. Briefly, the pMD18/PCV2-CL was PCR-amplified using primers P1 and P2 (Table 1) according to the instructions of the KOD-plus Kit (Toyobo, Ltd., Shanghai, China). The PCR products were then gel-purified and subsequently served as templates for fusion PCR using primers P3 and P4 (Table 1), which was used to insert the VP1 epitope gene (CTGACCAACGTG AGAGGCGATCTCCAAGTGCTGGCTCAGAAGGCGG CGAGGCCGCTGCCT) into ORF2, just before the stop codon. The fusion PCR product was then used to transform *Escherichia coli* strain Top10 cells according to the manufacturer's recommendations (Takara, Dalian, China). The obtained plasmid (pMD18-PCV2-CL-VP1) was subjected

Table 1 Primer sequences used in the construction and identification of recombinant virus

Primers	Position	Sequence (5′→3′)
P1	F1037	GGGTTTAAGTGGGGGGTCT
P2	R1033	ATGAATAATAAAAACAATTACGAAG
P3	F1017	TTGTTTTTATTATTCATTTAAGGCAGCGGCCTCGCCGCCTTCTGAGCCAGCACTTGGAGATCGCCTCTCACGTTGGTCAGGGGTTTAAGTGGGGGGTC
P4	R1056	AAGACCCCCCACTTAAACCCCTGACCAACGTGAGAGGCGATCTCCAAGTGCTGGCTCAGAAGGCGGCGAGGCCGCTGCCTTAAATGAATAATAAAAACAA
P5	F920	GTCGACGGAGGAAGGGGGCCAGTT
P6	R925	GTCGACTGTTCTGTAGCATTCTTCCA
P7	F959	CCCATGCCCTGAATTTCCATA
P8	R1311	TAAACTACTCCTCCCGCCATAC

to sequence analysis (BGI, Beijing, China). The sequence data were then compared with the data of the parental virus.

In vitro transfection

Plasmid pMD18-PCV2-CL-VP1 was digested with the *Sal*I restriction endonuclease and then the *Sal*I-purified fragments were self-ligated for 30 min at 16 °C using T4 DNA ligase (Takara). This DNA was then transfected into PK-15 cells (80–90 % confluency) in each well of a 24-well plate using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Empty plasmid-transfected PK-15 cells were included as a negative control. The transfected cells were cultured for 72 h at 37 °C in an atmosphere of 5 % CO_2 and then passaged as described previously (Liu et al. 2007). The genome of the recombinant virus was amplified with primers P5 and P6 (Table 1) and cloned into the pMD18-T plasmid for every two passages. Genomic sequencing of the recombinant virus was then performed by the dideoxy chain termination method using an automated sequencer (ALF Express).

Identification of recombinant virus

Immunoperoxidase monolayer assay (IPMA) plates for the parental and recombinant virus were prepared as described previously (Liu et al. 2004), and then tested with the PCV2-positive serum. The plates were stained and examined under a light microscope.

A pair of primers, P7 and P8 (Table 1), were designed to PCR-amplify the fragment containing the VP1 epitope gene. An Ex Taq DNA polymerase (Takara) was used to amplify fragments of the recombinant and parental viruses with the

following cycling program: 2 min at 94 °C, 35 cycles consisting of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, and a final extension step at 72 °C for 5 min. An amplified 413-base pair (bp) fragment was obtained using the recombinant virus as a template and a 353-bp fragment was obtained using the parental virus as a template.

To distinguish the recombinant virus from its parental virus, a capture ELISA was adapted from the methods of Huang et al. (2011b) with minor modifications. The HRP-anti-FMDV mAb (Ceditst) against the FMDV VP1 epitope was used to test the recombinant virus while the HRP-mAb-1D2 was used to test the recombinant and parental viruses. The colorimetric reaction was developed for 15 min by adding 100 μL of 3,3′,5,5′-tetramethylbenzidine substrate and the OD_{450} was measured using a spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Assays were performed with cultures of the recombinant virus, parental virus as a positive control, and mock-infected PK-15 cells as a negative control. The capture ELISA was performed in triplicate. The cut-off value for the ELISA was determined as the mean OD_{450} values of the negative control plus three standard deviations.

Morphology and propagation dynamics of the recombinant virus

To investigate the virus morphology, the recombinant and parental viruses was examined by immunoelectron microscopy as described previously (Huang et al. 2011b).

To investigate the virus propagation dynamics, the total virus (intracellular and extracellular virus) titers at 0, 12, 24, 36, 48, 60, and 72-h postinoculation (h.p.i.) were tested as described previously (Huang et al. 2010b; Liu et al. 2007). The propagation dynamics curve for the virus was plotted with virus growth time as the abscissa and the average $TCID_{50}$ of the different time points as the ordinate.

Infection and immunization of BALB/c mice with recombinant and parental viruses

Sixty-three 6-week-old female BALB/c mice were randomly divided into 3 groups (n=21 mice/group): group recPCV2-CL-VP1, group PCV2-CL, and group MEM, which were inoculated with recPCV2-CL-VP1, PCV2-CL and MEM (as a control), respectively. RecPCV2-CL-VP1 ($10^{4.0}$ $TCID_{50}$/mL), PCV2-CL ($10^{4.0}$ $TCID_{50}$/mL), and MEM was inoculated intranasally (50 μL) and intra-abdominally (1,000 μL), respectively. Three mice from each group were euthanized on 0, 7, 14, 21, 35, and 42 days post-inoculation (d.p.i.). The sera, hearts, livers, spleens, lungs, kidneys, and thymus were harvested for viral nucleic acids, antibodies, and pathological and histological analyses. The tissue specimens were fixed in formalin, embedded in paraffin, sliced, hematoxylin and eosin (HE) stained, and observed via microscopy.

Thirty-six 6-week-old female BALB/c mice were randomly divided into 9 groups (n=4 mice/group) and all were vaccinated every 2 weeks as the immune protocol shown in Table 2. The titers of parental and recombinant viruses used to prepare vaccines were $10^{4.3}$ TCID$_{50}$/mL, respectively. They were inactivated by methanol at 37 °C for 24 h. Mice were immunized subcutaneously with 0.5-mL vaccine. The IPMA and VP1-indirect ELISA were, respectively, applied to detect PCV2 and FMDV-VP1 antibodies.

The animal experiment was approved by Harbin Veterinary Research Institute, Chinese Academy of Agricultural Science and performed in accordance with animal ethics guidelines and approved protocols. The animal ethics committee approval number is Heilongjiang-SYXK-2006-032.

Serum antibody detection

IPMA and VP1-indirect ELISA were applied, respectively, to detect anti-PCV2 and anti-VP1 antibodies produced by infected animals. The IPMA was performed as described previously (Liu et al. 2004). The synthetic peptide (aa 131–170) containing the VP1 epitope was diluted to 4 µg/mL with 50 mM carbonate buffer (pH 9.6), coated on high-absorption 96-well ELISA plates, and then incubated overnight at 4 °C. The plate was then washed three times with 50 mM phosphate-buffered saline (PBS) containing 0.05 % Tween-20 at pH 7.2 (PBST). The plate was blocked with 2 % bovine serum albumin (BSA)-PBST, incubated at 37 °C for 1 h, and washed as described above. BSA-PBST-diluted mouse serum (1:50; 100 µL) was added to each well, incubated at 37 °C for 1 h, and then the plate was washed as described above. Diluted HRP-goat anti-mouse IgG (H+L) (dilution, 1:5000) was also added at 100 µL/well and incubated at 37 °C for 1 h. After

washing the plates three times, the colorimetric reaction was developed for 20 min by adding 100 µL 0.21 mg/mL of 2, 2-azino-di [3-ethylbenzthiazoline sulfonic acid] in 0.1 M citrate (pH 4.2) containing 0.003 % hydrogen peroxide (ABTS substrate). The reaction was stopped by adding 50 µL of 1 % NaF solution. The sera from FMDV O type vaccine and synthetic peptide of FMDV-VP1 groups were used as positive control and MEM-treated mouse sera as negative control. The ELISA was performed in triplicate and the cut-off value for the ELISA was determined as the mean OD$_{405 nm}$ value of the negative control plus three standard deviations.

Detection of virus nucleic acids

To investigate the viremia development and viral distribution in the tissues after incubation, DNA was extracted from BALB/c mouse sera (100 µL/mouse) and tissues (30 mg/tissue) using a DNA/RNA extraction kit (Takara). Viral DNA was extracted from sera collected at 0, 7, 14, 21, 28, 35, and 42 d.p.i. and analyzed by PCR. The viral loads in tissues were quantified by a real-time qPCR as described by Guo et al. (2012). The results were calculated as the mean of the logarithmic viral DNA copy number per 5 mg of tissue (Lg copies/5 mg).

Viral isolation

The mouse liver suspensions were diluted 20-fold and passed through a 0.45-µm filter and then a 0.22-µm filter. PK-15 cells were inoculated with the liver suspension concurrently at a dose of 10 %. After the cells formed a single-layer, D-glucose was added and the cells were cultured for 72 h. After three continuous passages, IPMA and PCR were, respectively, performed to identify the recombinant and parental viruses as described above.

Table 2 The immune program of mice

Groups	Immunogen	Adjuvant		
		The 1st vaccination	The 2nd vaccination	The 3rd vaccination
1	Inactivated recPCV2-CL-VP1	FCA[a]	FICA[b]	None[c]
2	Alive recPCV2-CL-VP1	FCA	FICA	None
3	Alive recPCV2-CL-VP1	None	None	None
4	Inactivated recPCV2-CL	FCA	FICA	None
5	Alive recPCV2-CL	FCA	FICA	None
6	Alive recPCV2-CL	None	None	None
7	FMDV O type vaccine	None	None	None
8	Synthetic peptide of FMDV-VP1	FCA	FICA	None
9	MEM	FCA	FICA	None

[a] Freund's adjuvant complete

[b] Freunds adjuvant incomplete

[c] Without adjuvant

DNA was extracted from the third passage using a DNA/RNA extraction kit (TaKaRa), which was then used as a template for PCR amplification with primers P7 and P8 (Table 1).

Statistical analysis

All data of IPMA, capture ELISA, indirect ELISA, and the qPCR were expressed as mean±SD. The IPMA, ELISA, and qPCR results were analyzed using one-way repeated measurement ANOVA followed by least significance difference (LSD) in the SAS system for Windows version 8.1 (SAS Institute Inc., Cary, NC, USA). The significance level was set at 0.05, and $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Rescue and antigenicity analysis of recombinant viruses

PK-15 cells were transfected with the infectious recombinant viral clone and viral antigens were confirmed by IPMA using PCV2-positive serum. Virally infected cells stained brownish-red, while control cells transfected in parallel with empty vectors did not stain (Fig. 1a). The recombinant viruses of 2, 4, 6, 8, and 10 passages were cloned into pMD18-T, and three independent positive clones of all generations were sequenced, and no mutation was revealed. The results verified that the recombinant viruses (recPCV2-CL-VP1) were successfully constructed and rescued.

Primers P7 and P8 were used to PCR-amplify the genomic DNA of recombinant and parental viruses, respectively. The PCR products of the recombinant virus had a length of 413 bp, which was longer than that of the parental virus (353 bp) because of the insertion of the FMDV-VP1 epitope (Fig. 1b). Mock-infected PK-15 cells were used as controls and revealed no bands indicative of the recombinant or parental viruses.

HRP-mAb-1D2 and HRP-anti-FMDV mAb were used to identify the recombinant and parental viruses and the results showed that HRP-mAb-1D2 reacted with both the recombinant and parental viruses, while HRP-anti-FMDV mAb only reacted with the recombinant virus; however, neither antibody reacted with the mock-infected PK-15 cells (Fig. 1c).

Morphology and propagation dynamics of the recombinant virus

The immune complex formed from the recombinant virus and the PCV2-positive serum were negatively stained and examined by electron microscopy, which revealed icosahedral viral particles with uniform shapes, diameters of approximately 17 nm, and similar to the parental virus (Fig. 2a).

The growth kinetic curves for the viruses were plotted with the growth time as the abscissa and the average $TCID_{50}$ value as the ordinate (Fig. 2b). Viral titers of the recombinant and parental viruses increased with time, but the proliferation ability of the recombinant virus was significantly inferior to that of parental virus.

### Characterization of the recombinant virus in mice

The PCR analysis showed that all tissues (heart, liver, spleen, lung, and kidney) and sera from the control mice were negative for PCV2. No antibodies specific for PCV2 or the FMDV-VP1 epitope were detected by PCV2-IPMA or VP1-indirect ELISA and no clinical symptoms, pathological changes, or microscopic lesions were observed in the control mice.

The IPMA was used to analyze the levels of PCV2-positive antibodies in the sera of BALB/c mice infected with the recombinant and parental viruses. PCV2-specific antibodies were detected 2 weeks post-infection in both of the infected groups, and the antibody titers gradually increased thereafter (Fig. 3a).

Viremia was then analyzed by PCR. No viremia was detected in three groups of recPCV2-CL-VP1, PCV2-CL, and MEM. Real-time PCR was used to detect viral nucleic acids in the heart, liver, spleen, lung, and kidney of the infected mice. As shown in Fig. 3b, recombinant and parental viral nucleic acids were detected in the heart, liver, spleen, lung, and kidney of the recombinant and parental virus infected mice at 7, 14, 21, 28, 35, and 42 d.p.i. (Fig. 3b). The viral loads in liver (7–42 d.p.i.) and kidney (7, 14, and 42 d.p.i.) were analyzed and the results showed that those in the group recPCV2-CL-VP1 were significantly higher than those in the group PCV2-CL ($p < 0.05$). The viral loads in the spleen (7, 14, 28, and 42 d.p.i.), lung (14–42 d.p.i.), and heart (7–42 d.p.i.) of group recPCV2-CL-VP1 were significantly lower than those in the group PCV2-CL ($p < 0.05$). There was no significant difference in the viral loads present in the kidney from the groups of recPCV2-CL-VP1 and PCV2-CL ($p > 0.05$) at 28 and 35 d.p.i., in the spleen from both groups ($p > 0.05$) at 35 d.p.i., and in the lung from both groups ($p > 0.05$) at 7 d.p.i.

BALB/c mice infected with either the recombinant or the parental virus did not develop any visible clinical symptoms or gross pathological changes. However, virally infected mice did display various levels of microscopic damage in the spleen, lung, kidney, and thymus (Fig. 4). Increased alveolar intervals and mild pulmonary hematoceles were detected in the lungs harvested at 35 d.p.i. from group recPCV2-CL-VP1 and typically increased alveolar intervals were found in the lungs harvested at 35 d.p.i. from group PCV2-CL. Hematoceles and some nuclei had been lost or even became pyknotic in the epithelial cells of the proximal convoluted tubules, the cytoplasm contained more acidophils in the kidneys of infected recPCV2-CL-VP1, and no apparent pathological lesions were found in the kidneys of mice infected PCV2-

Fig. 1 Characterization of the recombinant virus. **a** Antigen detection in the recPCV2-CL-VP1-infected PK-15 cells by IPMA. (*1*) RecPCV2-CL-VP1, (*2*) PCV2-CL, and (*3*) Mock. **b** Differentiation of the recPCV2-CL-VP1 from PCV2-CL by PCR. *Lane 1*: a 415-bp fragment amplified from the recPCV2-CL-VP1; *lane 2*: a 353-bp fragment amplified from PCV2-CL; *lane 3*: a mock-infected PK-15 control; *lane M*: DNA marker (TaKaRa). **c** Differentiation of the recPCV2-CL-VP1 from PCV2-CL by capture ELISA. For the capture ELISA, cultures of the recPCV2-CL-VP1, PCV2-CL, and mock-infected PK-15 cells were tested with the HRP-1D2-mAb and HRP-anti-FMDV mAb (Ceditst). The *error bars* represent the standard deviations
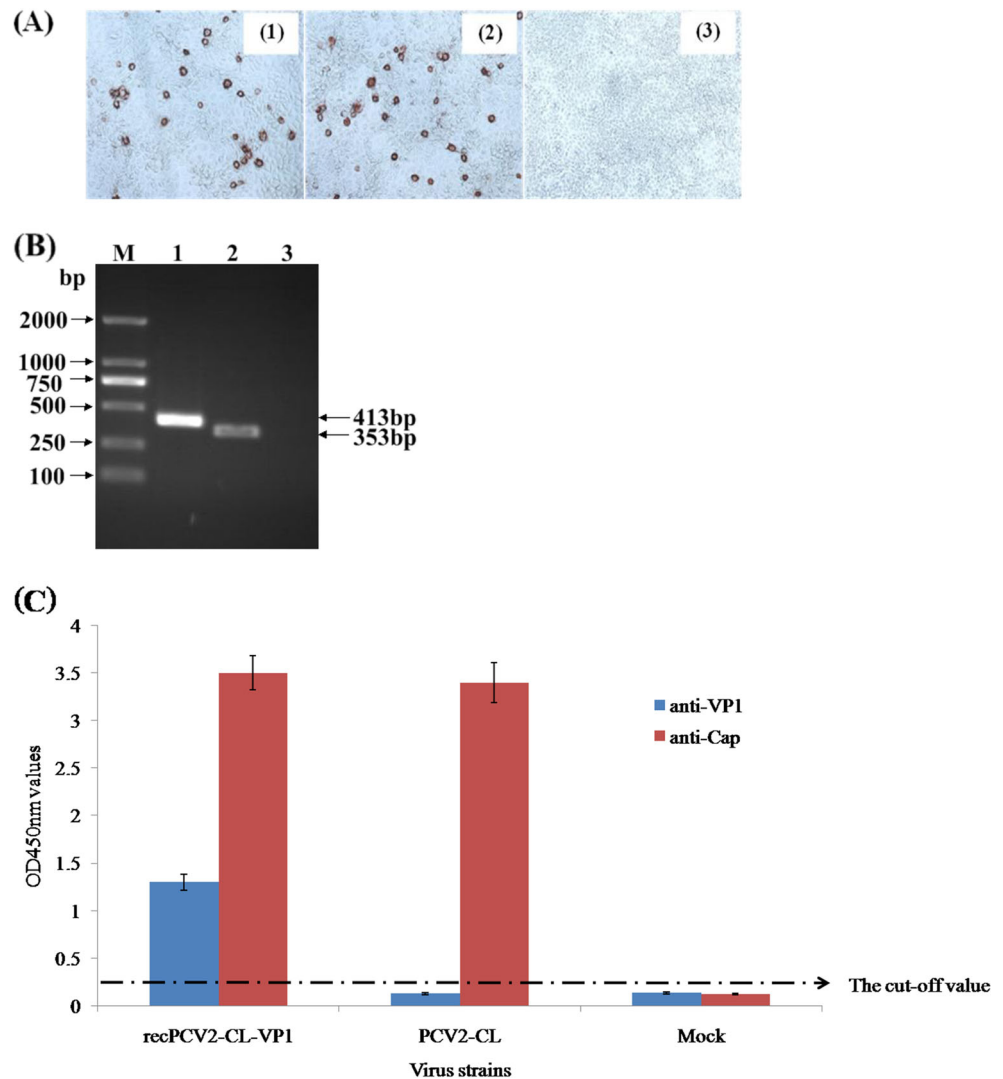
CL at 35 d.p.i.. In the spleens from group recPCV2-CL-VP1, the lymphocyte content decreased in both the red and white pulp and vacuoles of variable sizes appeared at 35 d.p.i., whereas trabecular venous congestion was observed at 35 d.p.i. from the PCV2-CL group. At 28 d.p.i., a mild decrease in lymphocyte content occurred in the thymuses from group recPCV2-CL-VP1 mice, but no major pathological lesions were detected in tissues from group PCV2-CL mice.

The IPMA results showed that viruses were successfully isolated from the liver of mice infected with the recombinant and parental viruses (Fig. 5a). Furthermore, the PCR results showed no viral cross-contamination between recombinant and parental viruses (Fig. 5b).

Antibody detection of immunized mice

IPMA and VP1-indirect ELISA were, respectively, used to detect PCV2 and FMDV-VP1 antibodies in mice vaccinated with the recombinant and parental viruses. The PCV2

antibody titer in mice vaccinated with the recombinant and parental viruses increased steadily with time; however, no significant differences were found (Fig. 6a). After twice vaccinations using the recombinant virus emulsified with Freund's adjuvant, FMDV-VP1 antibodies were detected, but not with recombinant viruses without Freund's adjuvant (Fig. 6b). FMDV-VP1 antibodies were not detected in the mice immunized with the parental virus (Fig. 6b).

Discussion

PCV2 is recognized as the infectious agent of PMWS, which reportedly causes severe economic losses to the pork industry. Consistently, the proportion of PCV2-positive pigs is very high worldwide. PCV2 damages lymphocytes and triggers immunosuppression (Allan et al. 2004), thereby intensifying the susceptibility of the infected animals to other pathogens,

Fig. 2 The morphological characteristics and growth kinetics of the recombinant virus. **a** Observations of the recPCV2-CL-VP1 and PCV2-CL via electron microscopy. (*1*) RecPCV2-CL-VP1 and (*2*) PCV2-CL. *Bar*=100 nm. **b** Growth kinetics of the recPCV2-CL-VP1. PK-15 cells were infected in parallel at a multiplicity of infection of 1 with passage 5 of the recPCV2-CL-V5 and PCV2-CL. At 0, 12, 24, 36, 48, 60, and 72 h.p.i., cells were harvested and virus titers were determined by IPMA. The results are presented as the mean values from three replicates of the experiment and expressed as log10 TCID$_{50}$/mL. The *error bars* represent the standard deviations
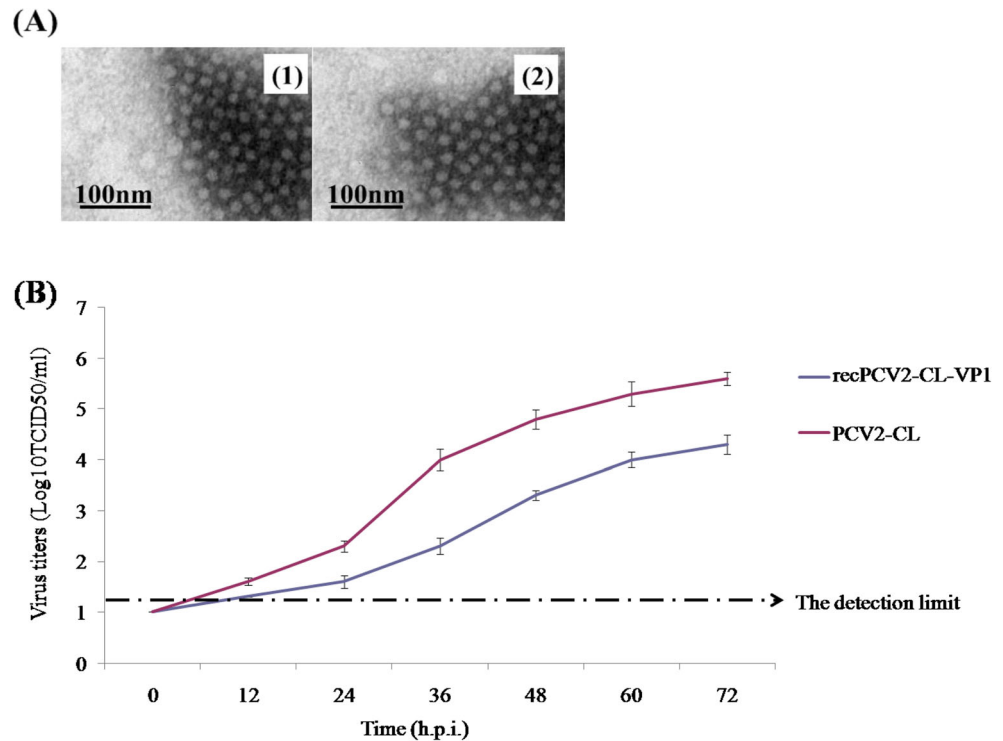
usually resulting in mixed infections (Ellis et al. 2004). Particularly, the mixed infections of FMDV, porcine reproductive, and respiratory syndrome virus, as well as classical swine fever virus and pseudorabies virus are the most devastating to swine populations (Allan et al. 2004; Ellis et al. 2004). Obviously, such mixed infections caused by several microorganisms are more difficult to control. Therefore, in the present study, the neutralizing antigenic epitope of FMDV-VP1 was successfully inserted into the Cap protein of PCV2, which was successfully expressed by the recombinant virus, thereby laying a foundation for the future development of a PCV2-FMDV bivalent vaccine.

A recombinant PCV2 expressing the FMDV-VP1 epitope was rescued and reacted with both PCV2-positive serum and neutralizing monoclonal PCV2 antibodies, just as its parental virus. We inferred that the insertion of the FMDV-VP1 epitope did not affect the initial antigenic characteristics and neutralizing activity of the Cap protein of the parental virus. This result suggests that the VP1 epitope did not completely overlay the major antigenic region, which can be explained by the following two reasons: first, the C-terminal is on the surface of the PCV2 virion and relatively far away from the highest protrusion of the Cap protein (Khayat et al. 2011); and second, the highest protrusion of the Cap protein was determined to be a major antigenic region for some neutralizing mAbs against PCV2 (Huang et al. 2011c; Saha et al. 2012). The reports of Beach et al. (2011) and Huang et al. (2011a) have also confirmed this explanation.

The recombinant and parental viruses can be distinguished by capture ELISA with the HRP-anti-FMDV mAb, which substantiated that the FMDV-VP1 epitope was present on the viral surface. This finding is logical because the C-terminal is on the surface of PCV2 virion as reported by Khayat et al. (2011) and results of Beach et al. (2011) and Huang et al. (2011b) have also shown that epitopes inserted into the C-terminal of the Cap protein were present on the surface of the recombinant virus.

The morphology of recombinant viruses observed via electron microscopy exhibited no visual difference from those of the parental virus. Thus, we conclude that the insertion of the FMDV-VP1 epitope had no influence on the assembly of the recombinant viral particles. However, the proliferation ability of the recombinant virus was significantly inferior to that of the parental virus. Huang et al. (2011b) indicated that insertion of the V5 epitope did not influence viral propagation. Therefore, we can presume that the exogenous epitopes inserted into the C-terminus of the Cap protein may have affected PCV2 propagation; however, this likely is dependent on the chemical characteristics of the particular foreign epitope.

In the present study, BALB/c mice were used to establish the recombinant and parental viral infection models. Although, it is already known that both BALB/c and Kunming mice are suitable for establishing PCV2 infection models (Kiupel et al. 2001; Liu et al. 2006; Shen et al. 2008; Li et al. 2010), in our study, the PCV2 injection failed to
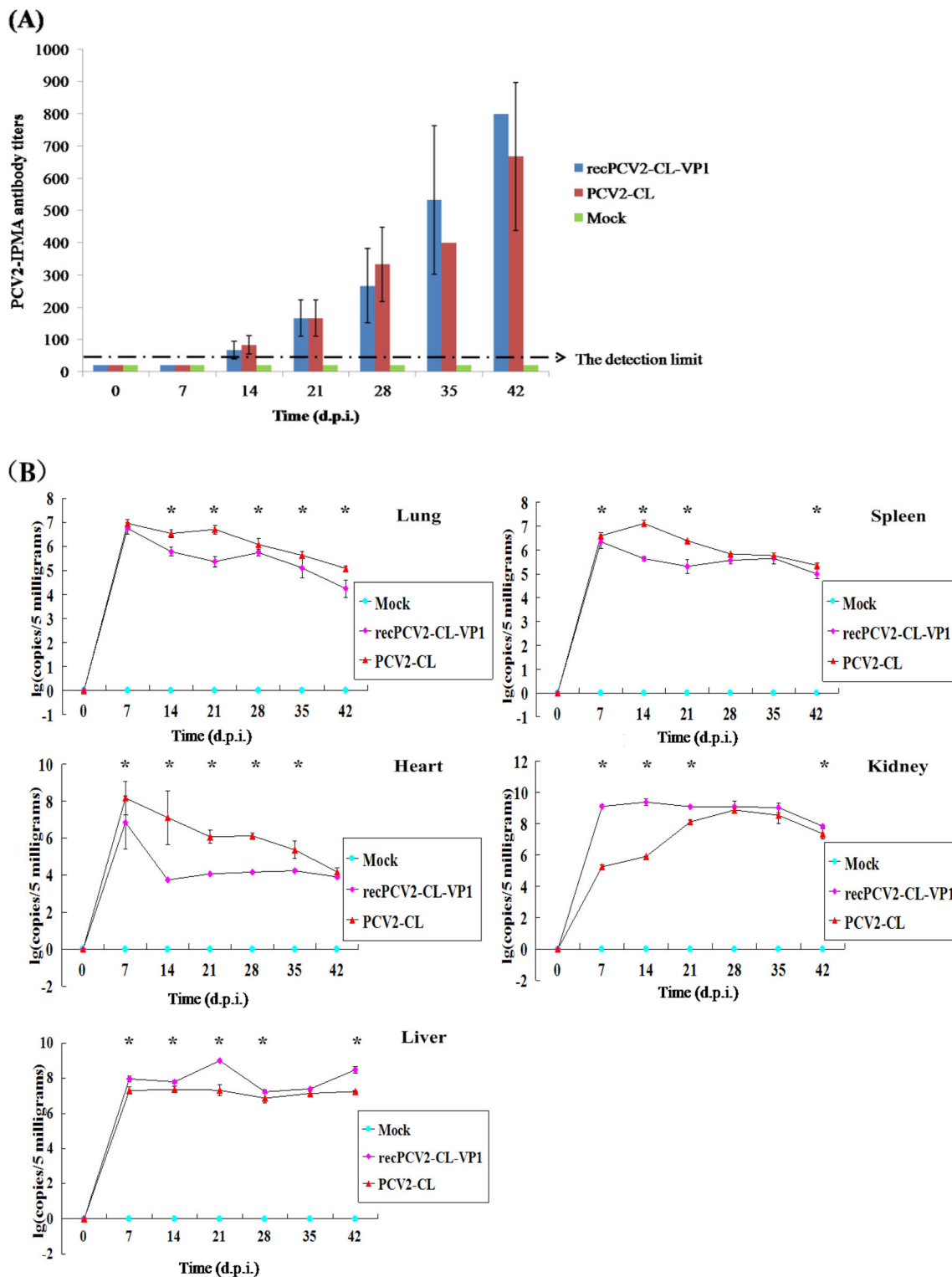
(A)

(B)

Fig. 3 The PCV2-IPMA antibody titers and viral load following viral infection. **a** The PCV2 antibody titers in the experimental mice. **b** Quantitative real-time PCR detection results of PCV2 viral loads in mice

induce apparent lesions, which might have been associated with the PCV2 strain as well as the dose and/or infection route. After intranasal and intra-abdominal infection of the BALB/c mice, nucleic acids of recPCV2-CL-VP1 and PCV2-CL were detected in various tissues, together with a high-level of anti-PCV2 antibodies. Both recPCV2-CL-VP1 and PCV2-
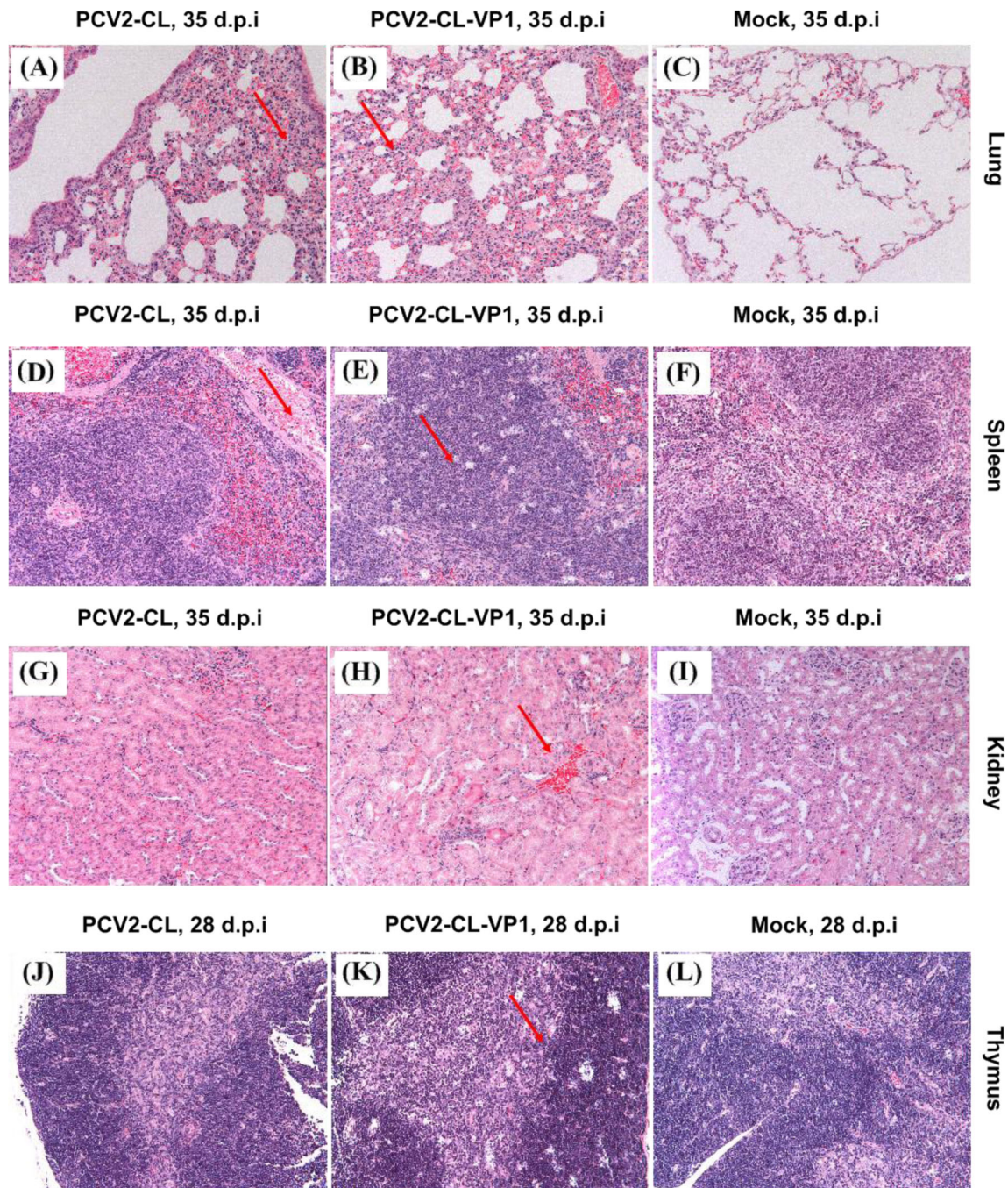
Fig. 4 Pathological sections of mice tissues following viral infection (HE staining; magnification, ×200). **a** Lung from a recPCV2-CL-infected mouse, 35 d.p.i. The distance between the alveoli widened. **b** Lung from a recPCV2-CL-VP1-infected mouse (35 d.p.i.). Slight congestion was observed and the distance between the alveoli widened. **c** Normal lung. **d** Spleen from a PCV2-CL-infected mouse (35 d.p.i.). Trabecular venous congestion was observed. **e** Spleen from a recPCV2-CL-VP1-infected mouse (35 d.p.i.). The lymphocyte content decreased and vacuoles were observed both in the red and white pulp. **f** Normal spleen. **g** Kidney from a PCV2-CL-infected mouse (35 d.p.i.). No obvious pathological significance was present. **h** Kidney from a recPCV2-CL-VP1-infected mouse (35 d.p.i.). Congestion, partial pyknosis of the proximal tubular epithelial cells, and enhancement of eosinophilic cytoplasm were observed. **i** Normal kidney. **j** Thymus from a PCV2-CL-infected mouse (28 d.p.i.). No obvious pathological changes were observed. **k** Thymus from a recPCV2-CL-VP1-infected mouse (28 d.p.i.). Lymphocyte reduction was observed. **l** Normal thymus

CL successfully replicated in the mice. The microscopic pathological lesions caused by the recombinant virus were more serious than those by the parental virus. We speculated that the inserted VP1 epitope might be responsible for the enhanced pathogenicity of the recombinant virus in mice. Fewer viral particles were isolated from the recPCV2-CL-VP1-infected
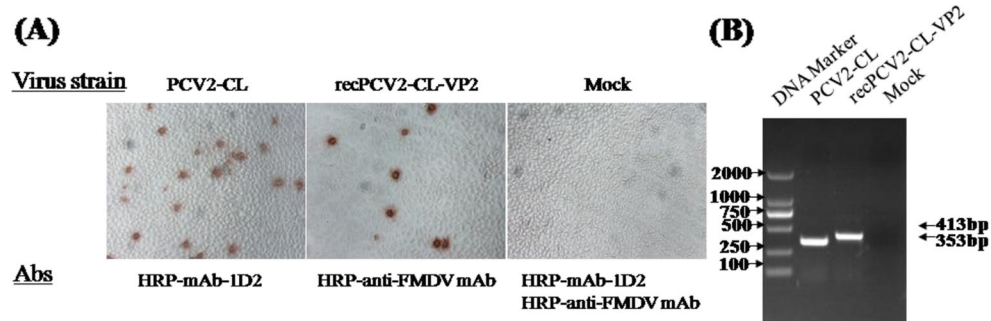
Fig. 5 Isolation and identification of recombinant and parental viruses from mice liver. **a** Staining the recombinant and parental viruses isolated from mice livers by IPMA. The HRP-anti-FMDV mAb (Ceditst) against the FMDV VP1 epitope was used to test the recombinant virus while the HRP-mAb-1D2 was used to test the parental viruses. **b** Identification of the recombinant and parental viruses isolated from mice livers by PCR

mice tissues than from the PCV2-CL-infected tissues. This result could be due to the lower proliferative ability of the recPCV2-CL-VP1 compared to PCV2-CL in PK-15 cells.

The FMDV-VP1 antibodies in mice vaccinated by activated recPCV2-CL-VP1 with Freund's adjuvant and mice vaccinated by inactivated recPCV2-CL-VP1 with Freund's adjuvant began to turn positive after the second vaccination. However, the FMDV-VP1 antibody was not detected in mice vaccinated with activated recPCV2-CL-VP1 only. One possible explanation might be that adjuvant is necessary to enhance
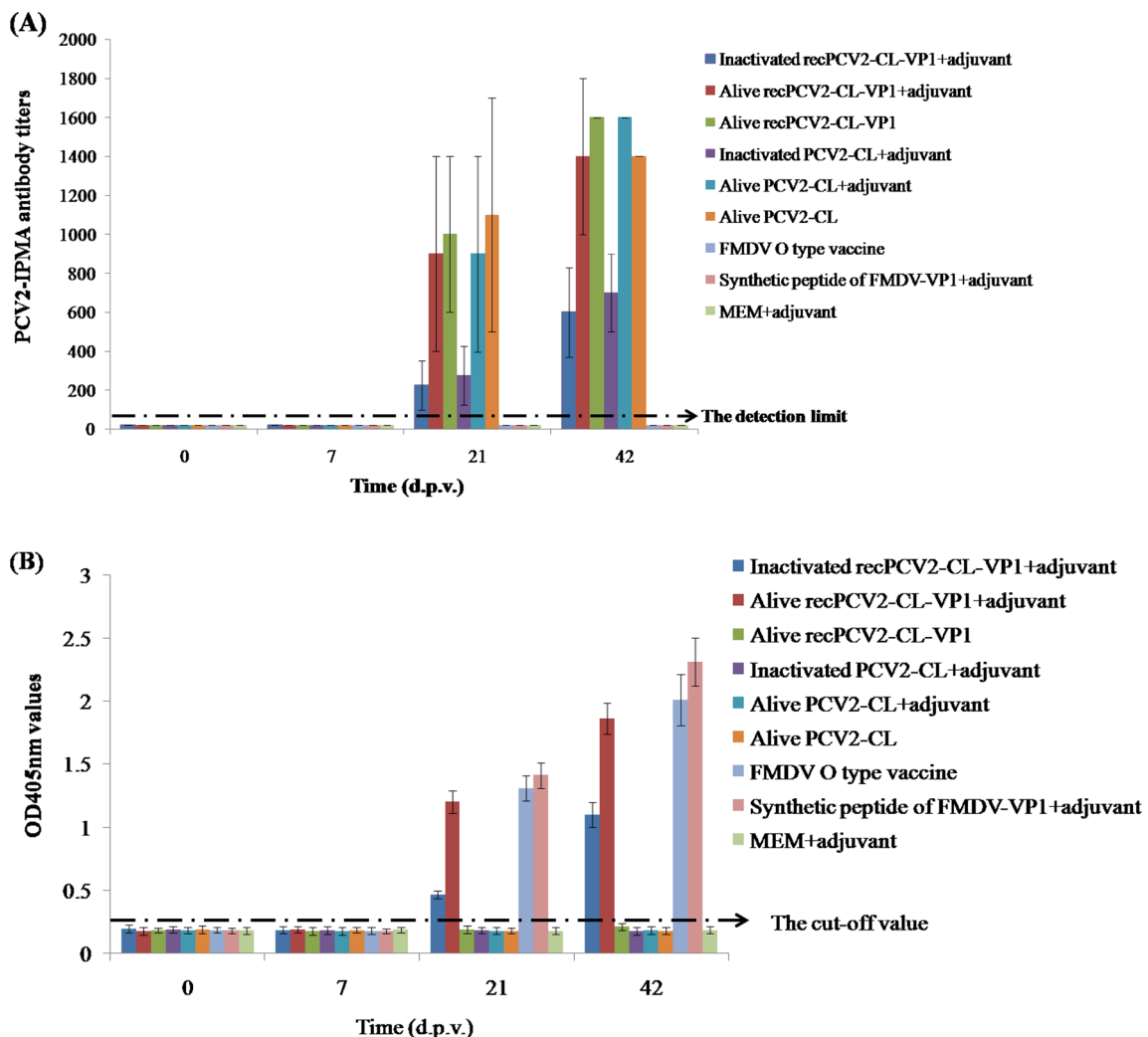

Fig. 6 The VP1 and Cap antibodies in mice. **a** The Cap antibodies in mice. **b** VP1 antibodies in mice

the immune response against the VP1 epitope. However, once vaccination with recPCV2-CL-VP1 induced a higher titer of PCV2 antibodies, while the antibodies against the neutralizing epitope of FMDV-VP1 were only detected after the use of Freund's adjuvant. This might be explained by the following three reasons: first, the recombinant virus only contained one antigen epitope of FMDV, which did not induce a strong immune response in the mice; second, the folding and packaging of the PCV2-Cap protein might have affected the full exposure of epitope; and third, the viral antigen of FMDV type O used in this study had a lower immunogenicity compared to other strains.

Herein, we successfully rescued recombinant PCV2 expressing the neutralizing VP1 epitope (aa 141–160) of FMDV type O. The morphological features of the recPCV2-CL-VP1 strain were similar to those of its parental strain (PCV2-CL). Additionally, propagation of the recombinant virus was stable in PK-15 cells. Subsequent analyses revealed that both of the recombinant and parental viruses replicated in the mice caused various pathological changes, and induced a high level of anti-PCV2 antibodies. More importantly, the recombinant virus emulsified with Freund's adjuvant induced antibodies against the neutralizing VP1 epitope region (aa 141–160) of FMDV. In conclusion, the present study provides a basis for the development of a PCV2 marker vaccine and a PCV2-FMDV bivalent vaccine.

Conflict of interest The authors declare that they have no conflict of interest.

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