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Construction and characterization of the infectious clone of porcine parvovirus carrying genetic marker

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ABSTRACT

Porcine parvovirus (PPV) is one of the major pathogens that bring about reproductive failure of pregnant sows. However, the study of the pathogenesis mechanism is circumscribed due to the lack of efficient genetic manipulation method. Infectious clone is a powerful tool for further studying the genetic mechanisms of PPV. In the present study, the gene fragment (157–4812) of PPV was amplified by PPV China isolate strain as a template, and PPV DNA fragments (1–182) forming Y-structure within in 5′ end and (4788–5074) forming U-structure in 3′ end were synthesized. And then, the above three fragments were inserted into plasmid pKQLL to congregate a PPV full-length recombinant plasmid by means of In-Fusion cloning technology. After the successful sequencing identification of the recombinant plasmid, the *Eco*R I restriction site was brought out as a genetic marker by nonsense mutation (A3058 T) to produce plasmid Y-PPV, which was transfected into PK-15 cells for rescue of virus. The rescued viral particles were observed under transmission electron microscopy, and the sequencing analysis showed that Y-PPV could stably carry the genetic marker. It could be seen that Y-PPV has similar replicate capability and pathogenicity as the wild-type parental PPV strain by cellular and animal experiments. These results confirmed that Y-PPV maintain similar biological characteristics with wild-type parental PPV strain. Infectious clone could be a valuable tool for studying the individual genes of PPV and applications in gene deletion or live vector vaccines.

1. Introduction

Porcine Parvovirus (PPV) is a pathogen that can lead to reproductive failure in swine, characterized by embryonic resorption, abortion and stillbirths (Zhou et al., 2017). So far, vaccination has been widely used to prevent PPV infection, but the spread of the disease is not checked completely (Jozwik et al., 2009).

Porcine Parvovirus is a small non-enveloped virus with single stranded linear DNA genomes which is about 5 kb length (Lin et al., 2013). The genome is flanked by inverted terminal repeats (ITRs) sequence, which are necessary to form a palindromic hairpin structure as the replication origin of Parvovirus (Wang et al., 2016). The genome of PPV contains two major open reading frames, ORF1 and ORF2; the ORF1 encoded nonstructural protein NS1, NS2 and NS3. NS1 is the most major non-structural protein and plays a vital role in viral replication, with nuclease, helicase, and gene transactivation activities. In addition, NS1 protein can induce cell cycle arrest (Xu et al., 2017), activate host innate immunity, and inhibit the exogenous type I interferon signaling (Wu et al., 2016). NS2 protein can interact with nuclear export factor (CRM1), which is required for efficient nuclear egress of progeny viral (Bodendorf et al., 1999; Eichwald et al., 2002). NS3 is the smallest non-structural protein, which is involved in viral replication and production of progeny virus (Huang et al., 2014). The ORF2 encodes capsid proteins VP1 and VP2, VP2 is further cleaved into VP3 in the endosomes, which is considered to produce the necessary space for the VP1 N terminus externalization (Farr et al., 2006; Ros et al., 2002; Sanchez-Martinez et al., 2012). The VP1 sequence contains the entire VP2 sequence and the N-terminal extension 150-amino-acid called VP1 up, VP1 and VP2 are necessary for the generation of infectious progeny virus (Fernandes et al., 2011). However, the function of PPV structural proteins and non-structural proteins in the process of pathogenic mechanism is still unclear. Hence, it is necessary to construct a cell-adapted cDNA clone of PPV that can be used as a convenient platform for studying structural protein and no-structural protein roles in PPV

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infection and pathogenesis.

Currently, most studies about parvovirus focus on Murine Minute virus (MVM), Muscovy duck and Human Parvovirus (Merchlinsky et al., 1983; Wang et al., 2015, 2016; Yen et al., 2015; Zhi et al., 2006, 2004), while limited research on porcine parvovirus. An infectious clone constructed from the avirulent vaccine strain, NADL-2, which has been identified as no-pathogenic to susceptible animals (Vasudevacharya et al., 1990). However, there are significant sequence differences between the epidemic strain and highly pathogenic Kresse strain (Fernandes et al., 2011; Meszaros et al., 2017), which largely limit the application of the infectious clone in the study of PPV abortion and pathogenesis. In the present study, the full-length genome of wild-type parental PPV was cloned and analyzed. And the efficient and stable infectious clone of Y-PPV carrying a genetic marker was constructed. The biological characteristics of the rescued Y-PPV were compared with wild-type parental PPV in *vitro* and in *vivo,* and the results revealed that Y-PPV retained a level of biological characteristics similar to that of the wild-type parental PPV strain, which will provide a valuable tool for the study of molecular pathogenic of PPV and the application of gene deletion or live vector vaccines.

2. Materials and methods

2.1. Cell lines and virus

Porcine Kidney (PK-15) cells (ATCC) were cultured in Dulbecco-modified minimal essential medium (DMEM) (Gebico, USA) supplemented with 10% Fetal Bovine Serum (Si Jiqing, China). The PK-15 cells were incubated at 37 °C under 5% CO_2 and used for propagating the PPV. Wild-type parental PPV China isolate strain (Genbank: MK993540) adapted in PK-15 cells was used for the construction of infectious cDNA clone.

2.2. Cloning of full-length coding region amplification of PPV viral DNA and sequence analysis

Viral DNA was extracted from PPV infected PK-15 cells as the PCR template. The PPV coding region genome was cloned by Prime STAR PCR Enzyme (Takara, China) using PPV-F and PPV-R primers (Table 1). The PCR product was cloned into the pMD-18 T vector, which was then transformed *Escherichia coli* strain *Stbl* 3 and sequenced. For comparative studies, the complete genome sequences of wild-type parental PPV strain was compared with that of Genbank by using the MegAlign program packaged in the Lasergene package 5.0.

2.3. Construct carrying a genetic marker full-length infectious clone plasmid DNA

As previously described (Ma et al., 2010). As the ITR hairpin structure in this sequence is basically reverse complementary, it is difficult to clone it by conventional PCR methods. Therefore, the palindrome sequences were sent to the company for synthesis (Huada, China), with *Bam*H I and *Xba* I restriction sites at the both ends. And the intermediate sequences (157–4812) was amplified by primers PPV-F and PPV-R, using viral genomic DNA as template. These fragments were composed together to form PPV full length genome through In-fusion cloning technique, which was then linked to low copy plasmid pKQLL to construct PPV full length infect clone. Further, non-sense mutations were obtained using overlap PCR using P-F/P-R 1 and P-F1/P-R (Table 1), and then the fragments were connected to pKQLL-PPV to form PPV full length infect clone, named Y-PPV.

Table 1
Primers used in the study.

Prime	Prime sequence	Amplification size
P-F	AAAAAAGAGGCGGGAAAAAAAGAGG	4600 bp
P-R	TGTTTTTTGGGGATAATTGGTATACAG	
P-F	AAAAAAGAGGCGGGAAAAAAAGAGG	2900 bp
P-R1	CATGAATTCTTTTGTATGTTTCGTGTTC	
P-F1	GAACACGAAACATACAAAAGAATTCATG	1700 bp
P-R	TGTTTTTTGGGGATAATTGGTATACAG	
P-F2	CAACAATGGCTAGCTATATGCA	1420 bp
P-R2	CTAGTGCGACCATTAAGCTTGC	

2.4. Rescuing of infectious virus

In order to estimate whether genomic clone could be infectious, the plasmid Y-PPV was transfected into PK-15 cells with a cell density of 70%. Infectivity was determined by the development of cytopathic effect (CPE), and viral particles were observed under transmission electron.

2.5. Immuno-fluorescence assay and confocal imaging

For immuno-fluorescence (IF), the assay was performed as previously described (Ganaie et al., 2017). The cells were incubated to the primary antibodies 3C9 (a mouse anti-PPV capsid-specific monoclonal antibody; CRL-17; ATCC) for 1 h at room temperature. After further washings, the cells were incubated with anti FITC-mouse secondary antibodies for 1 h at room temperature in the dark. After the final washings, the cover glasses were removed from the wells and were fixed onto a slide using confocal microscopy (LECIA TCS SP8, Germany).

2.6. Western blotting assay

The cell lysates was denatured by heating were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes, which was blocked in PBS buffer with 5% non-fat dry milk at room temperature for 1 h, and incubated with anti-PPV capsid-specific monoclonal antibody 3C9 followed by HRP-conjugated secondary antibodies. Luminescent signals were detected using enhanced chemiluminescence (ECL) reagent.

2.7. Quantification of death cells by flow cytometry

The cell mortality was detected using the 7-AAD strain assay kit (Biolegend, 420404) according to the manufacturer's protocol. Rescued Y-PPV, wild-type parental PPV infected and uninfected cells were digested by trypsinization without EDTA and washed three times with ice-cold PBS. The precipitate after centrifugation was resuspended in 150 µl binding buffer, and then 5 µl 7-AAD was added. The mixture was then incubated for 20 min at room temperature in the dark. The stained cells were analyzed by flow cytometry (Becton Dickinson Accuri C6, US).

2.8. Caspase activity assay

As previously described (Zhang et al., 2015). Caspases colorimetric assay kits (Beyotime Biotech, China) were used to measure the caspase-3, caspase-8 and caspase-9 activity, according to the manufacture's recommendations, the cells were treated with lysis buffer, and protein concentration was measured using BCA Protein Assay Reagent (Beyotime Biotech, China). Then 200 µg lysates of each sample were loaded into microplates and incubated with each caspase substrate at 37 °C for 4 h, the absorbance values of samples were measured at 405 nm in microplate spectrophotometer (Infinite 200 PRO NanoQuant, Tecan, Switzerland). Caspase-8 inhibitor Z-IETD-FMK (catalog number C1230, Sigma, St. Louis, MO, USA), Caspase-9 inhibitor Z-LEHD-FMK (catalog number C1355, Sigma) were dissolved in dimethyl sulfoxide (DMSO,

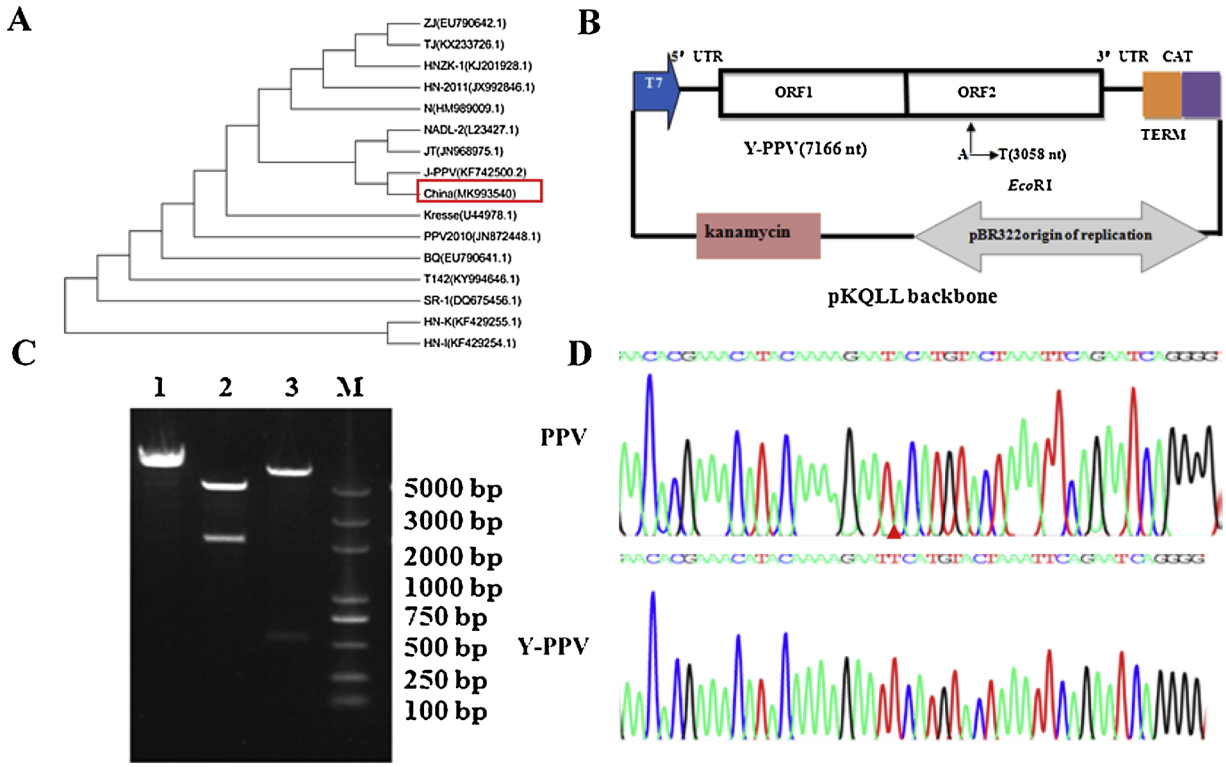

Fig. 1. Construction and identification of full-length infectious cloning plasmid of Y-PPV. **(A)** The numbers at the forks indicate the bootstrap values (1000 replicates). Strains used in this study are indicated by filled triangles, red box marked as PPV China isolate strain. **(B)** Schematic map of Y-PPV plasmid DNA. UTR, noncoding region; TERM, TonB terminator. **(C)** Restriction enzyme digestion of Y-PPV. M: DNA markers.1: untreated plasmid; 2: Double digestion with *Xba* I and *Bam*H I. 3: Single digestion with *Eco*R I. **(D)** Sequences of a Y-PPV and wild-type parental PPV. The T-to-A mutation in theVP1 gene of PPV is indicated by the frame. Red triangle marked as nonsense mutation position (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

catalog number D2650, Sigma). PK-15 cells were treated for 1 h and were washed for three times with PBS to remove inhibitors, cells were then infected, in the presence of the inhibitor, for 2 h and were washed again. And the caspase-3, caspase-8 and caspase-9 activity were measured.

2.9. Identification of the genetic marker in rescued virus

The genetic marker stability experiment was performed according to previously described methods (Chen et al., 2017). The PK-15 cells were incubated with rescued Y-PPV for 36 h, and its cell culture was collected for freeze-thaw three times, and the cell culture was incubated a fresh PK-15 cells and culture 36 h. This process proceeded to the 15th generation in the same way. The DNA of 5th, 10th and 15th generation of Y-PPV was extracted and detected by PCR using primers P-F2 and P-R 2, and the PCR purified products were identified by *Eco*R I digestion and sequencing.

2.10. Rescuing virus replication and pathogenicity properties

To estimate whether the genetic marker affected the characterization of the rescued Y-PPV, PK-15 cells with a density of approximately 50% were infected with the rescued Y-PPV and wild-type parental PPV strain (1MOI). And the replication level, virulence and apoptotic were measured at different time points of post infection.

2.11. Animal experiment

Handling of animals and experimental procedures were performed in accordance with the guide lines for caring of laboratory animals of Northwest A&F University. All animal experiments were approved by the institutional committee of Northwest A&F University. All of the gilts were free of PPV, PRRSV, PRV and PCV2. The nine gilts were equally divided into control, rescued Y-PPV and wild-type parental PPV infection groups. The pregnant gilts were exposed intranasally to 10 ml (10^7 $TCID_{50}$/ml) or 10 ml of DMEM cell culture medium at Day 22 of gestation and designated as 0 day post-infection (0 d p.i.). The blood was collected from ear vein every 7 days after PPV infection and control group. And then the PPV antibody levels were determined by diagnostic kit for antibodies to porcine parvovirus (Approval Number, CFC002AA, Ai Ruide Biotechniques, Ltd., Jiangsu, China). The progesterone levels were determined in the serum by radio-immuno assay using iodine [^{125}I]-progesterone RIA kit (Approval Number S10950183, Jinding Biotechniques, Ltd., Tianjin, China). At 35 dp.i, all sows were euthanized and the heart, liver, spleen, lung, kidney, uterus, oophoron and brain were collected. To test the viral load in different tissues, PPV were isolated from difference tissues in the infected sows as described by Miao et al (Miao et al., 2009). In brief, these tissues were grind using a pestle and mortar, and then resuspended in 1 ml sterile PBS. After freezing and thawing for three times and centrifugation for 10 min at 1000 × g, the supernatant was passed through a 0.22 μm filter. A 100 μl sample was extracted viral DNA using the phenol-chloroform method. The viral load of organs was determined by real-time PCR. Each sample was tested three times, and the mean cycle threshold of each sample was recorded. Besides, the uterus and oophoron were fixed and hematoxylin-eosin staining was performed to observe the pathological changes of the tissue.

2.12. Statistical analysis

Data were shown as means ± SEM (SD) values representing of three independent experiments. Each experiment was carried out in

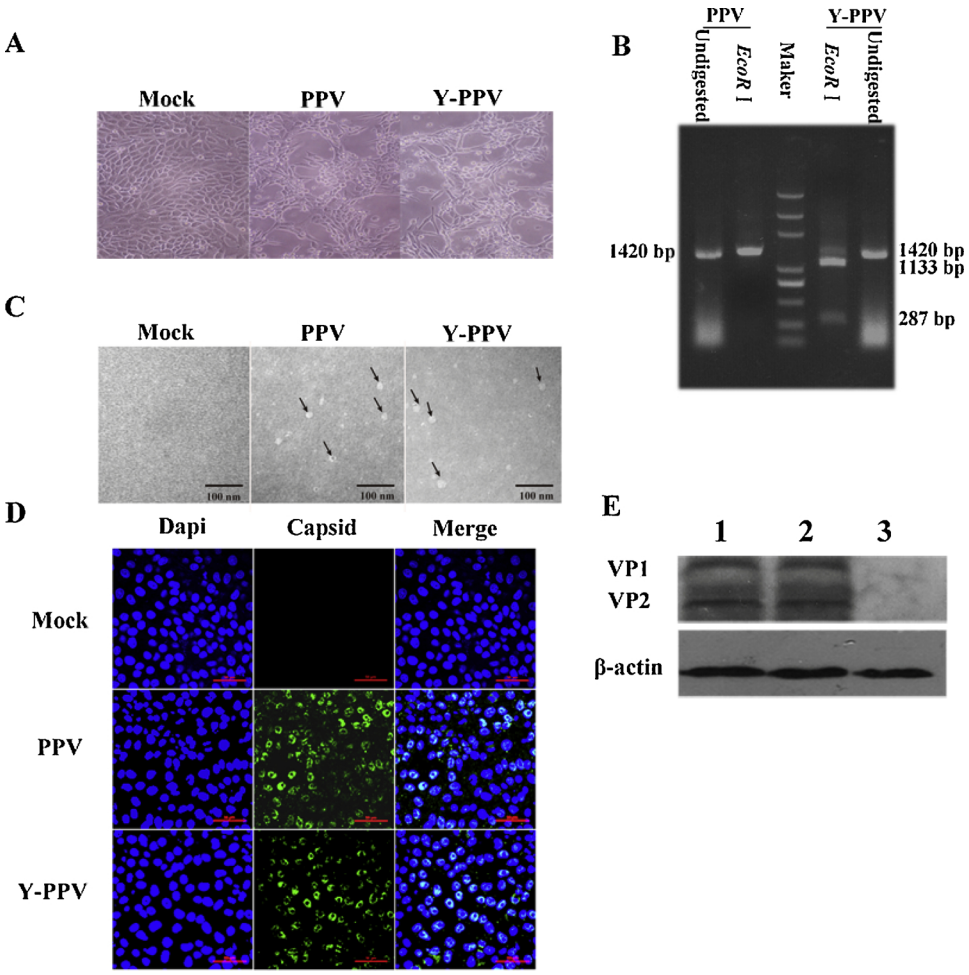

Fig. 2. Identification of rescued Y-PPV virus assay. **(A)** PPV-specific cytopathic effect (CPE) of rescued virus. PK-15 cells culture were collected at 36 h after transfected with Y-PPV plasmid, and continuously infected for 5 generations. Cells were observed under fluorescence microscope with natural light (×100). **(B)** Identification of the genetic marker in the rescued Y-PPV virus. The *Eco*R I restriction enzyme site was introduced into the recombinant plasmid to create a genetic marker to distinguish the rescued virus from the parental virus. Two 1420 bp fragments from parental PPV and rescued Y-PPV with primers P-F2/P-R 2, and the Y-PPV could produce a 287 bp fragment by *Eco*R I digestion. **(C)** Observation of rescued Y-PPV virus under transmission electron microscopy. PK-15 cells were infected with Y-PPV or wild-type parental PPV at MOI 1 for 36 h and culture supernatant were absorbed to copper mesh, and staining with phosphotungstic acid, then viral particles morphology was observed under transmission electron microscopy(×120,000). Black arrows indicate PPV viral particles. **(D)** Immunofluorescence analysis of rescued Y-PPV virus. The PK-15 cells were infected with Y-PPV or wild-type parental PPV. At 36 h p.i, the infected cells were stained by anti-PPV capsid 3C9 monoclonal antibody and observed under confocal microscopy. (E) Expression of VP1 and VP2 in Y-PPV infected cells. The PK-15 cells infected with Y-PPV or wild-type parental PPV were detected by western blotting using anti-PPV capsid 3C9 monoclonal antibody at 36 hp.i. 1:PPV; 2:Y-PPV; 3:Mock.

triplicate. Statistical comparison of the results was analyzed by one way analysis of variance (ANOVA). A value of $P < 0.05$ was considered as significance.

3. Results

3.1. Construction of full-length cDNA clone plasmid and sequence analysis of PPV China isolate strain

The PPV coding sequence was amplified by primers P-F and P-R for sequencing and comparision with different strains that published in the NCBI database. The phylogenetic analysis showed that wild-type parental PPV strain had the closest genetic relationship with J-PPV (Fig. 1A). Using the genomic DNA of wild-type parental PPV strain as template, a PPV DNA fragment (157–4812) was amplified by PCR. In addition, a PPV DNA fragment (1-182) in 5′ terminus Y structure and a PPV DNA fragment (4788–5074) in 3′ terminus U structure sequence were synthesized directly. Lastly, these three fragments were cloned into plasmid pKQLL to assemble a complete PPV full-length recombinant plasmid by In-Fusion cloning technology. After sequencing identification, we further bought in an *Eco*R I restriction site as genetic marker via nonsense mutation (A3058 T) and named Y-PPV. Fig.1B showed the structure schematic diagrams of Y-PPV plasmid DNA. Restriction enzyme analysis and sequencing of Y-PPV showed that a genetic marker was successfully introduced at 3058 of the PPV genomic DNA (Fig.1 C, D).

3.2. Rescuing of infectious Y-PPV

The infectious cloning plasmid Y-PPV containing genetic marker was transfected into PK-15 cells and continuously infected for 15 generations. Cell infection experiment displayed that Y-PPV infection could induce obvious cytopathic effect (Fig. 2A). The primer P-F2/P-R 2 was used to amplify the DNA fragment containing *Eco*R I genetic marker in Y-PPV, but not in wild-type parental PPV. Restriction enzyme analysis revealed that the amplified fragment of Y-PPV infected cells could produce a 287 bp fragment by *Eco*R I digestion, but the amplified fragment of wild-type parental PPV infected cells could not (Fig. 2B). The virions generated by Y-PPV infection were the similar with their wild-type parental PPV strain under electron microscopy (Fig. 2C). Furthermore, indirect immuno-fluorescence and western blotting assays showed that PPV capsid protein could be detected in the culture supernatant of cells infected with progeny Y-PPV (Fig. 2D, E). The above results indicated that PPV could be rescued from the cells transfected with Y-PPV plasmids, and the rescued Y-PPV has similar infection characteristics to the wild-type parental PPV.

3.3. Rescuing virus stability and characterization

In order to determine the genetic marker stability of the rescued Y-PPV, Y-PPV continuously infected for 15 generations. The analysis of restriction enzyme showed that fragments amplified by Y-PPV infect cells Using P-F2/P-R 2 as primers could produce a 287 bp fragment by *Eco*R I digestion at 5th, 10th and 15th generations, while the fragments amplified from the wild-type PPV infected cells could not (Fig. 3A). The Y-PPV exhibited similar replicate capacity and pathogenicity as wild-

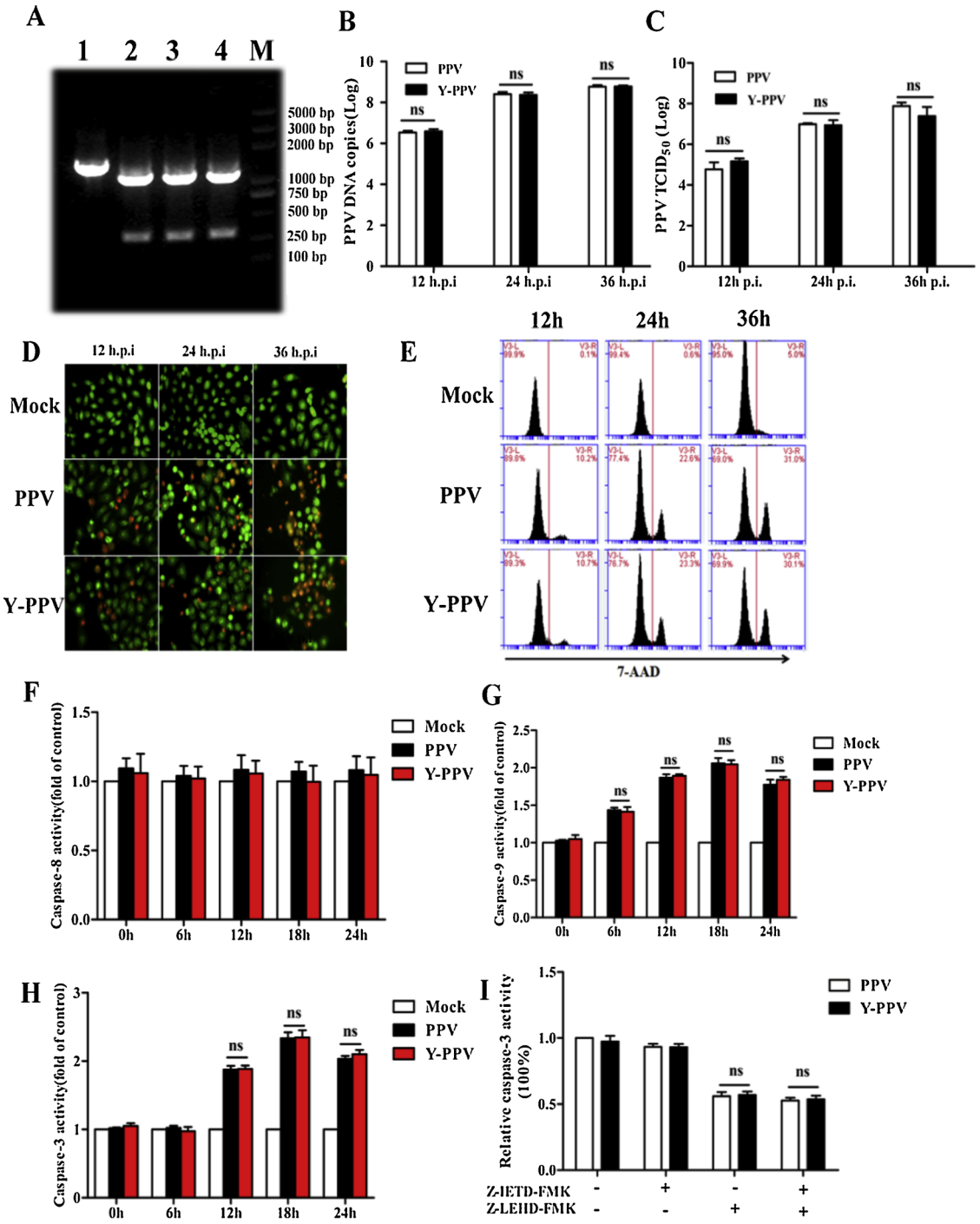

Fig. 3. Characterization assay of rescued Y-PPV. **(A)** Rescue virus genetic marker genetic stability; M: DNA markers.1: Wild-type parental PPV DNA fragment treat with *Eco*R I digestion; 2-4: Rescued Y-PPV 5th, 10th and 15th DNA fragments were treated with *Eco*R I digestion. (B) Detection of rescued Y-PPV and wild-type parental PPV DNA copy numbers (copies/100 μl) at infection different time points. (C) Detection of rescued Y-PPV and wild-type parental PPV $TCID_{50}$ at infection different time points. $TCID_{50}$ indicate per milliliter viral load. (D) AO/EB staining assay detection of rescued Y-PPV and wild-type parental PPV induced apoptosis. (E) Flow Cytometry detection of rescued Y-PPV and wild-type parental PPV-induced cell death rate. (F) The enzymatic activities of caspase-8 in rescued Y-PPV and wild-type parental PPV-infected cells. (G) The enzymatic activities of caspase-9 in rescued Y-PPV and wild-type parental PPV-infected cells. (H) The enzymatic activities of caspase-3 in rescued Y-PPV and wild-type parental PPV infected cells. (I) The effect of initiator caspase-8 and caspase-9 on the activation of caspase-3. Results are recorded by means ± SD. ns: no significant difference.

type parental PPV at different time points after infection (Fig. 3B, C). In addition, AO/EB staining showed that the rescued Y-PPV could induce cell death effect at different time points post-infection (Fig. 3D). Furthermore, flow cytometry showed that the rescued Y-PPV induced similar cell death rate as wild-type parental PPV at different time points post-infection (Fig. 3E).

Previous work has revealed that wild-type parental PPV infection could induces apoptosis through activation caspase-9 and caspase-3 not caspase-8 pathway (Zhang et al., 2015), we further identified the activity of caspases in the cells infected with the rescued Y-PPV, the results showed that the rescued Y-PPV induced similar activation features in caspase-9, caspase-3 and caspase-8 as wild-type parental PPV

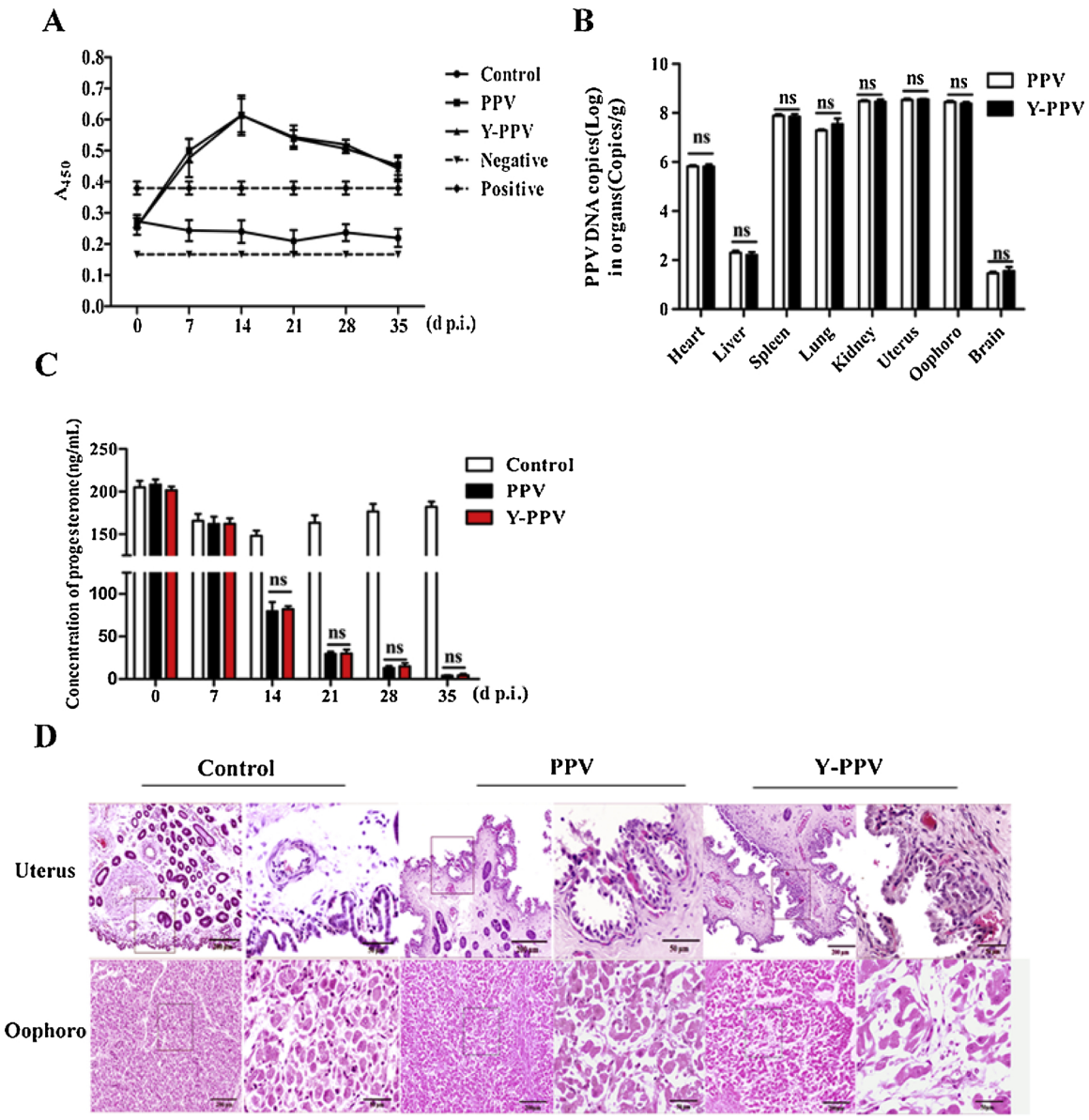

Fig. 4. Characterization of rescued Y-PPV *in vivo* assay. (A) Effects of Y-PPV or PPV-infected on PPV IgG antibody levels. After PPV infection, the PPV IgG antibody levels became positively. (B) The PPV loads in the different organs of infected sows. The *x*-axis indicates the examined organs, the *y*-axis represents viral loads as the logarithm of PPV DNA copies in the different organs. (C) Rescued Y-PPV and wild-type parental PPV inoculation reduces the levels of progesterone. The pregnant gilts were intranasally exposed to 10 ml (10^7 $TCID_{50}$/ml) or 10 ml of DMEM cell culture medium (Control). Then the serum progesterone levels were determined by RIA. (D) Morphology observation of Hematoxylin-eosin (HE) staining uterus and oophoro in wild-type parental PPV, Y-PPV and control groups.

(Fig. 3F, G, H). To further determine the contribution of caspase-8 or caspase-9 to activate caspase-3, we analyzed the inhibitory efficacy of caspase-8 or caspase-9 inhibitor in caspase-3 activity in cells infected with the rescued Y-PPV. As shown in Fig. 3I, the activity of caspase-3 was significantly inhibited by caspase-9 inhibitor, but was not by caspase-8 inhibitor treatment, which were similar as the characteristics of wild-type parental PPV. The above results indicate that rescued Y-PPV is similar to wild-type parental PPV in replication capacity and pathogenicity characteristics.

3.4. Animal experiment

After PPV infection, the PPV IgG antibody levels became positively, the PPV IgG antibody levels of rescued Y-PPV and wild-type parental PPV infection group were significantly higher than that of control group after 7 d p.i., but there was no significantly difference between these two groups in PPV IgG antibody level (Fig. 4A). Rescued Y-PPV and wild-type parental PPV DNA were detected in heart, liver, spleen, lung, kidney, uterus, oophoro and brain after infection of pregnant sows with and rescued Y-PPV and wild-type parental PPV. Besides, the viral loads of kidney, lung, uterus and oophoro were significantly higher than that in other tissue, but there was no significant difference between Y-PPV and wild-type parental PPV-infected sows in the viral load of same tissues (Fig. 4B). Furthermore, the effects of Y-PPV infection on luteal function, serum progesterone levels were measured. Circulating progesterone levels maintained in a relatively normal level in control group. However, the circulating progesterone levels of rescued Y-PPV and wild-type parental PPV infection group were significantly lower than that of control group after 14 d p.i., but no significantly difference appeared between these two groups (Fig. 4C). Lastly, HE staining results showed that both the rescued Y-PPV and wild-type parental PPV could induce obvious tissue lesions in uterus and oophoro (Fig. 4D).

4. Discussion

PPV infection mainly cause reproductive failure of pregnant swine, including infertility, embryonic death, stillbirth and fetal mummification in pregnant sows, it has brought huge economic losses for animal aquaculture in the entire world (Antonis et al., 2006).

PPV can bind to sialic acids on the cell surface receptor and used both clathrin mediated endocytosis and macropinocytosis pathways to gain access into cells (Boisvert et al., 2010). In recent study suggested that PPV infection can through the different approach induce cell death, such as induce mitochondria apoptotic mediated ROS accumulation and p53 activition signaling pathway (Zhang et al., 2015; Zhao et al., 2016), activates NF-κB signaling pathways and induce inflammatory cytokine production (Cao et al., 2017; Zhou et al., 2017), but it is not clear which protein plays a role in these processes, since lack of effective mutant strains. Infect clone was widely used as a convenient platform for studies disease vaccines and therapeutics, which contribute to study virus pathogenic mechanism, virus-host interaction and vaccine development (Tsetsarkin et al., 2016).

The PPV China isolate strain full-length gene sequence was cloned and analyzed, as shown in the phylogenetic tree, PPV China isolate strain and the J-PPV strain tended to group together in a distinct phylogenetic clade. The J-PPV stain (Genbank: KF742500.2) was PPV circulating in 2014 from China (Wang et al., 2014).

Reverse genetics is a powerful and efficient tool to research gene-function and viral pathogenic mechanism (Deng et al., 2012; Weger-Lucarelli et al., 2017). B19 V effective replication is necessary in the presence of adenovirus helper genes or adenovirus(Guan et al., 2009), and the adenovirus helper function is not necessary for pIHBoV1 and ADV-G replication in sensitive cells (Huang et al., 2012; Xi et al., 2017). In this study, for the first time, stable carrying a genetic marker full-length DNA infectious clone was constructed using *in vitro* In-Fusion PCR (Fig.1), and the infectious clone Y-PPV was rescued through transfection into PK-15 cells without any adenovirus helper genes or adenovirus.

The rescued Y-PPV and wild-type parental PPV had identical characteristics in terms of virus particle formation under electron microscopy (Fig. 2C). Furthermore, indirect immuno-fluorescence and western blotting assay manifested that PPV capsid protein was able to detect in the culture supernatant of cells infected with progeny Y-PPV (Fig. 2D, E). The above results indicate that PPV could be rescued from the cells transfected with Y-PPV plasmids, and that rescued Y-PPV is similar to wild-type parental PPV in infectious characteristics.

In order to determine the genetic marker stability of the rescued Y-PPV, the rescued Y-PPV continuously infected for 15 generations. Restriction enzyme analysis showed that the fragment amplified from the Y-PPV-infected cells was able to produce a 287 bp fragment by *Eco*R I digestion at 5th, 10th and 15th generations, while the fragment amplified from the wild-type PPV infected cells was not able to produce (Fig. 3A). Besides, virions generated by Y-PPV infection possessed similar replicate capacity and pathogenicity as wild-type parental PPV at infection different time points (Fig. 3B, C). In addition, the rescued Y-PPV could induce cell death effect and cell death rate by stained with AO/EB and flow cytometry (Fig. 3D, E). Lastly, we found that rescued Y-PPV induced similar features of activated caspase-9 and caspase-3 increased as early as 12 h p.i., but caspase-8 activity was not observed change in this study compare to wild-type parental PPV (Fig. 3F, G, H, I). In a word, rescued Y-PPV and wild-type parental PPV infection exhibited same features in triggering PK-15 cells apoptosis.

Besides, rescued Y-PPV and wild-type parental PPV showed a same infection characteristics in the tissue tropism, and there is no significant difference between the Y-PPV and wild-type parental PPV in the viral load of same tissues (Fig. 4B). PPV Chinese isolate BQ strain was found in the heart, lung, spleen, kidney and endometrium in sow (Miao et al., 2009), which is agree with our experiment results. Y-PPV infection can significantly decrease circulating progesterone levels of the blood, which is also consistent with the results observed by Zhang et al in PPV-infected animals (Zhang et al., 2018). Cellular and animal experiments manifested that Y-PPV possessed similar replicate capability and pathogenicity as the wild-type parental PPV. These results demonstrate that Y-PPV does not change the biological characteristics of wild-type parental PPV.

In summary, we constructed a carrying stable genetic marker PPV infectious clone, Y-PPV, and cellular and animal experiments showed that rescued Y-PPV possessed similar replicate capability and pathogenicity as the wild-type parental PPV. This work provides a convenient and available tool for further studying of the pathogenic mechanism of PPV, and will greatly facilitate studying the function of individual gene of PPV and gene modification for PPV live vector vaccines development.

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