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Development of a reverse genetics system for a feline panleukopenia virus

Nan Cheng[1,2] · Yongkun Zhao[1,4] · Qiuxue Han[1,6] · Weijiao Zhang[1] · Ji Xi[3] · Yongle Yu[3] · Hualei Wang[1,4] · Guohua Li[1,5] · Yuwei Gao[1,4] · Songtao Yang[1,4] · Weiquan Liu[3,7] · Xianzhu Xia[1,4]

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Abstract

Feline panleukopenia virus (FPV) infects cats and can be fatal to kittens. There is evidence that canine parvovirus originated from FPV, which makes FPV important in studies of the family *Parvoviridae*. In the present study, the entire genome of FPV strain HH-1/86 was converted into a full-length infectious clone (pFPV). The FPV strain HH-1/86 has a 5123-nt single stranded DNA genome with a Y-shaped inverted 3′ terminal repeat (ITR) and a U-shaped inverted 5′ ITR. Feline kidney cells (F81) were transfected with the pFPV clone which contained a genetic marker, and a rescued virus was obtained (rFPV). The rFPV was identified by its cytopathic effects, indirect immunofluorescence, growth curve analysis, western blot assay and hemagglutination, and was indistinguishable from the parent virus. The FPV infectious clone will facilitate the study of pathogenicity and viral replication of FPV and the inter-species transmission of parvoviruses.

Keywords Feline panleukopenia virus · Infectious clone · Rescue virus · Biological properties

Introduction

Feline panleukopenia virus (FPV) is a member of the genus *Protoparvovirus* in the family *Parvoviridae* [1]. FPV mainly infects cats and causes acute gastroenteritis and leukopenia, which is often fatal to kittens [2]. Other felids, such as tigers, lions, and jaguars, can also be infected by FPV [3].

The FPV genome is a single stranded DNA of 5123 nt with inverted terminal repeats (ITRs) at both ends, and encodes four proteins [4]. Previous reports have indicated that both ITRs are important for viral replication [5]. The viral genome contains two open reading frames (ORFs). ORF-L expresses two non-structural proteins, NS1 and NS2, while ORF-R expresses structural proteins VP-1 and VP-2 [6, 7]. VP-2 is the major capsid protein of FPV and

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Nan Cheng and Yongkun Zhao contributed equally to the results of this study.

✉ Songtao Yang
yst62041@163.com

✉ Weiquan Liu
weiquan8@cau.edu.cn

✉ Xianzhu Xia
xiaxzh@cae.cn

[1] Key Laboratory of Jilin Province for Zoonosis Prevention and Control, Institute of Military Veterinary, Academy of Military Medical Science, 666 Liuying Xi Road, Changchun 130122, China

[2] College of Veterinary Medicine, China Agricultural University, Beijing, China

[3] College of Biological Sciences, China Agricultural University, Beijing, China

[4] Jiangsu Co-innovation Centre for Prevention and Control of Important Animal Infectious Disease and Zoonosis, Yangzhou, China

[5] College of Animal Science and Technology, Shihezi University, Shihezi, China

[6] College of Veterinary Medicine, Northeast Agricultural University, Harbin, China

[7] State Key Laboratory of Agro-Biotechnology, Department of Biochemistry and Molecular Biology, College of Biological Sciences, China Agricultural University, No. 2 Yuanmingyuan West Road, Haidian District, Beijing 100193, China

determines the antigenicity, pathogenicity and host range of the virus [8].

Several other viruses in the parvovirus genus are similar to FPV, including minute virus of mice (MVM), mink enteritis virus (MEV), and canine parvovirus type 2 (CPV-2), all of which are named after their natural hosts [9]. Parvoviruses have many similar antigenic features, and the sequences of their capsid proteins are highly homologous. However, hemagglutination inhibition and virus neutralization tests show that while there is common antigenicity between FPV, MEV, and CPV-2 [10], these viruses are very different in terms of host cell specificity and when analyzed with monoclonal antibodies [11–13]. MEV replicates in feline cells in vitro but does not infect dogs and cats in vivo, whereas FPV replicates in feline cells in vitro and infects cats and mink in vivo.

FPV was first identified in the 1920s and named in 1939. MEV was identified in 1947 and isolated in 1952, whereas CPV-2, the original type of CPV, which also infects cats, was identified in 1976 [14, 15]. Two variant types of CPV, CPV-2a, and CPV-2b, were identified in 1978 and 1986 and cause serious infections in dogs worldwide [16]. A third variant (CPV-2c) was first identified in Italy in 2000 and rapidly emerged in canines in Europe, Asia and America [17–19]. Based on their times of emergence and highly homologous sequences [20], it is apparent that both MEV and CPV-2 originated from FPV [21], which suggests that FPV could infect different species of carnivores after only a few mutations [22]. Furthermore, a case of FPV infection in monkeys was recently reported in Beijing, China. The infection caused hemorrhagic diarrhea, fever, and anorexia and resulted in a fatal outcome with a mortality of 50% [23]. This case confirmed the reality of inter-species transmission of FPV from felines to non-human primates. Considering this finding, a FPV reverse genetics system would be a valuable tool to study the in vitro replication of the virus and its pathogenicity to determine the molecular mechanisms underlying inter-species transmission and host ranges of parvoviruses.

In the present study, the full-length genome of an FPV strain, HH-1/86, was converted into a full-length infectious clone (pFPV) with an *Xho*I genetic marker by applying PCR-based tools, including synthesized oligonucleotides and the In-Fusion^{TM} assembly system [24]. The virus was rescued following transfection of feline kidney cells with pFPV.

Materials and methods

Viruses and cells

The FPV strain HH-1/86 was isolated from a dead jaguar in Shanghai in 1986 by our laboratory group. Feline F81 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Cloning of the full-length genomic sequence

DNA from FPV strain HH-1/86 was isolated using the Viral DNA Extraction Kit (Axygen) according to the manufacturer's instructions. The specific primers and synthesized oligonucleotides used for amplifying the ends of the FPV full-length genome were based on FPV strains MG132167A and FPV-b (GenBank accession numbers: KP769859 and M38246) (Table 1).

The specific primers for the U structure at the 5′ end and the Y structure at the 3′ end of the viral DNA are shown in Table 1 as U1-F/R, U2-F/R, Y1-F/R and Y2-F/R. The ITR regions were amplified using Prime STAR HS DNA polymerase (Takara). For amplification, 2 μL viral DNA

Table 1 Primers used for amplification of the FPV genome

Name	Sequence (5′–3′)	Position	Length (bp)
Y1-F	ATCATTCTTTAGAACCAACTGACCAAG	1–27	69
Y1-R	GCAGCGCGCGCAGCGCGCGTCAT	47–69	
Y2-F	GCTGCGCGCGCTGCCTAC	56–73	123
Y2-R	AACCACGCCCACAATTAGCCCG	166–187	
M1-F	TTGTGTGTTTAAACTTGGGC	134–153	1596
M1-R	GTTGTCATAATTACTGGAGTTGG	1707–1729	
M2-F	GGAAGTAAGCAAATTGAACC	1689–1709	1688
M2-R	AAACCCAATGTCTCAGATCTC	3356–3376	
M3-F	CTGTTTCAGAATCTGCTACTC	3241–3261	1678
M3-R	GGTTAGTTCACCTTATAGACAG	4897–4918	
U1-F	TAATGTATGTTGTTATGGTGTGG	479–4814	237
U1-R	CTACGCGGTCTGGTTGATTAAGC	5006–5029	
U2-F	GCGGTCTGGTTGATTAAGC	5027–5046	96
U2-R	AAGTATCAATCTGTCTTTAAGGGG	5100–5123	

was mixed with 12.5 μL 2 × Prime STAR GC buffer, 2 μL dNTPs (2.5 μM), 2 μL primers (Y1-F, Y2-F, U1-F, and U2-F) (10 pmol/μL), and 5 μL ddH_2O. The mixture was first boiled for 5 min then chilled immediately on ice for 10 min, then 1 μL Prime STAR HS DNA polymerase was added, heated at 72 °C for 3 min and mixed with one of the primers (Y1-R, Y2-R, U1-R, or U2-R). The ITR regions were amplified with the following conditions: 98 °C for 5 min; 35 cycles of 95 °C for 10 s, 60 °C for 10 s, 72 °C for 10 s, and finally 72 °C for 10 min. The PCR products were separated by agarose gel electrophoresis and purified using the DNA Gel Extraction Kit (Axygen).

The specific primers for the middle regions of FPV are shown in Table 1 as M1-F/R, M2-F/R and M3-F/R. The middle region was amplified using Prime STAR GXL DNA polymerase (Takara) with the following cycling conditions: 98 °C for 5 min; 35 cycles of 98 °C for 10 s, 55 °C for 10 s, 68 °C for 20 s; and, finally 68 °C for 10 min.

Construction of the full-length infectious clone

To generate the infectious clone, a strategy involving overlap PCR, inclusion of synthesized oligonucleotides and the In-Fusion™ assembly system was adopted. The specific primers used for overlap PCR to combine fragments M1, M2 and M3 are shown in Table 2 as O1-F, O2-R, and O3-R. The ITRs region of the genome was obtained by synthesizing two complementary oligonucleotides, I and II. Fragment I and the 3′ end of the genome were generated as a synthesized 192 bp sequence and ligated into the pBluescript II SK (+) vector. Fragment II was generated as a synthesized 145 bp sequence and also cloned into the pBluescript II SK (+) vector, generating the plasmid p (3′ + 5′), which was renamed pE. Next, a pair of primers (Infusion-F and Infusion-R) was designed using M1 + M2 + M3 as templates and used to amplify a 4784 bp middle sequence of FPV. Finally linearized pE and the middle sequence were joined using an In-Fusion™ HD enzyme premix (Takara) to construct recombinant plasmid pFPV. The entire strategy used to construct the full-length infectious clone is shown in Fig. 2.

Nucleotide 2981 was changed from A to G by site-directed mutagenesis, a non-coding change forming a unique *Xho*I site, to exclude the possibility that the rescued virus was contaminated. Specific 750-bp DNA fragments were obtained by PCR. The specific primers used are shown in Table 2 as JF and JR.

DNA transfection and virus recovery

Six-well plates were seeded with F81 cells and cultured in DMEM (Gibco) supplemented with 10% FBS with incubation at 37 °C in 5% CO_2. On the following day, the culture medium was replaced with Opti-MEM (Gibco), and the cells were transfected with 3.5 μg pFPV mixed with 7.5 μL Lipofectamine 3000 (Invitrogen). The cells were washed twice with Opti-MEM and incubated at 37 °C in 5% CO_2 for 4 days in Opti-MEM supplemented with 10% FBS. Supernatant media were collected, and the cells were infected at a multiplicity of infection (MOI) of 0.1 rFPV in 2 mL DMEM. After incubation at 37 °C in 5% CO_2 for 4 days, the cultures were passaged ten times, with supernatants collected at each passage to be tested for virus isolation.

Table 2 Primers using for construction of full-length plasmid pFPV

Name	Sequence (5′–3′)	Position
A2981G-F	GGAAATCACAGCAAACTCGAGCAGACTTGT	2963–2992
A2981G-R	CGAGTTTGCTGTGATTTCCACCCATCC	2955–2981
JF	CACCAATGAGTGATGGAGCA	2782–2802
JR	CCTGTAGCAAATTCATCACCTG	3556–3577
Infusion-F	GGGCGTGGTTAAAGGTATAAAAGACAAA	178–205
Infusion-R	ATACTTATGGTAAGGTTAGTTCACCTTATAGACA	4898–4931
O1-F	TTGTGTGTTTAAACTTGGGC	134–153
O2-R	AAACCCAATGTCTCAGATCTC	3356–3376
O3-R	GGTTAGTTCACCTTATAGACAG	4897–4918
I	GGTACCATCATTCTTTAGAACCAACTGACCAAGTTCACG TACGTATGACGTGATGACGCGCGCTGCGCGCGCTGCCTA CGGCAGTCACACGTCATACGTACGCTCCTTGGTCAGTTG GTTCTAAAGAATGATAGGCGGTTTGTGTGTTTAAACTTG GGCGGGAAAAGGTGGCGGGCTAATTGTGGGCGTGGT	1–192
II	TAAAGGCCTTACCATAAGTATCAATCTGTCTTTAAGGGG GGGGTGGGTGGGAGATACACAACATCAGTAGACTGACTG GCCTGGTTGGTTGCTCTGCTTAATCAACCAGACCGCTAC GCGGTCTGGTTGATTAAGCGCTGGATCC	4917–5048

Virus titration and indirect immunofluorescence assay

F81 cells were infected with $rFPV_5$ (the fifth passage of the virus) or FPV strain HH-1/86. Staining was performed at 72 h by adding 0.05 mL anti-FPV VP2 and FITC-rabbit anti-mouse IgG antibodies successively to each drained well for 1 h of incubation at 37 °C. After 2× washing with PBS the cells were examined for FITC fluorescence by microscopy.

Virus samples (0.1 mL) were prepared, in quadruplicate, in 96-well microplates as 10-fold serial dilutions to 10^{-8} in DMEM (2% FBS). F81 cell suspensions (0.05 mL) diluted in growth medium to a concentration of 4×10^4 cells/mL were added to each well, and the plates were incubated for 48 h at 37 °C in a humidified incubator with 5% CO_2. After 48 h incubation, the cells were stained by addition of 0.05 mL FITC-conjugated mouse-anti-FPV VP2 antibody to each well. After a 1 h incubation at 37 °C, each plate was washed 2x with PBS, then incubated with FITC-rabbit anti-mouse IgG antibody at 37 °C for 1 h. Fluorescence was assessed microscopically, and endpoints were taken as the highest dilutions at which half of the wells (2/4) still exhibited fluorescence. The virus titers were calculated as 50% tissue culture infectious doses ($TCID_{50}$).

Western blot analysis

F81 cells were infected or transfected with $rFPV_5$, $rFPV_{10}$, FPV HH-1/86, and pFPV. The cells were collected and analyzed by SDS-12% PAGE before transferring the proteins onto a polyvinylidene difluoride (PVDF) membrane (Whatman) for Western blotting with canine anti-FPV VP2 and horseradish peroxidase (HRP)-conjugated goat-anti-dog IgG antibodies (Sigma).

Hemagglutination assay (HA)

Serial two-fold dilutions of the samples were prepared with 15 mM PBS (pH 6.5) in 96-well V plates, and 25 µL PBS and 50 µL 1% (v/v) piglet erythrocytes containing 0.5% rabbit serum were then added. Results of the HA test were read after 1 h of incubation at 4 °C and endpoint titers were taken as the highest dilution showing at least partial agglutination.

Results

Sequence analysis

The complete sequence of the genome of FPV strain HH-1/86 was amplified by PCR and uploaded to GenBank (GenBank accession number: KX900570). The FPV strain HH-1/86 was with more than 98.0% homology with the other FPV strains, CPV strains, and MEV strains (Table 3), apart from the strain CPV-193.

Construction of the full-length infectious clone and virus recovery

Synthesized oligonucleotides and specific primers (Table 2) were based on genomic sequences of FPV strain HH-1/86, according to the previously described strategy (Fig. 1a). The middle region of the virus was separated into three parts and combined by overlap PCR amplification (Fig. 1b). Finally the middle sequence (4784 bp) and linearized pE were joined using an In-Fusion™ HD enzyme premix to construct recombinant plasmid pFPV (Fig. 1c). The rescued virus ($rFPV_0$) produced by transfection was infectious. Typical FPV cytopathic effects (CPE) developed by day 3 post-transfection. The cells were partly detached and show a less density because the FPV infection. The cells also form a network like CPE which prove the infection of parvovirus, while mock-transfected cells remained normal (Fig. 2a).

Western blotting and genetic marker identification

VP2 expression by the recombinant virus was also confirmed by Western blotting. Viruses (HH-1/86, $rFPV_5$ and $rFPV_{10}$) were harvested at 48 h post-infection. Extracts of rFPV and HH 1/86-infected cells were stained with canine polyclonal antibody against VP2. Western blotting analysis of all three preparations showed protein expression at 67 kDa (Fig. 2b). By *Xho*I digestion, the 0.75-kb PCR product containing the genetic marker of $rFPV_5$ and $rFPV_{10}$ could be cleaved into 0.55-kb and 0.20-kb fragments, but the PCR product of the parental virus (HH-1/86) was not cleaved (Fig. 2c).

Indirect immunofluorescence assay, HA results and growth curve of the rescued virus

To determine VP2 protein expression in F81 cells, the cells were examined using indirect immunofluorescence. F81 cells were grown in 96-well plates and infected with HH-1/86 or $rFPV_5$. Fluorescence was observed by confocal

Table 3 Comparison of amino acid sequences between FPV HH-1/86 and other parvovirus strains (%)

Strain	GenBank accession number	NS1 protein	VP2 protein
FPV CU-4	P24840.1	99.1	99.8
CPV-N	M19296.1	99.6	98.3
CPV-193	AY742932.1	99.6	97.9
Abashiri	D00765.1	99.4	99.5
MEV/LN-10	HQ094567.2	99.4	99.7

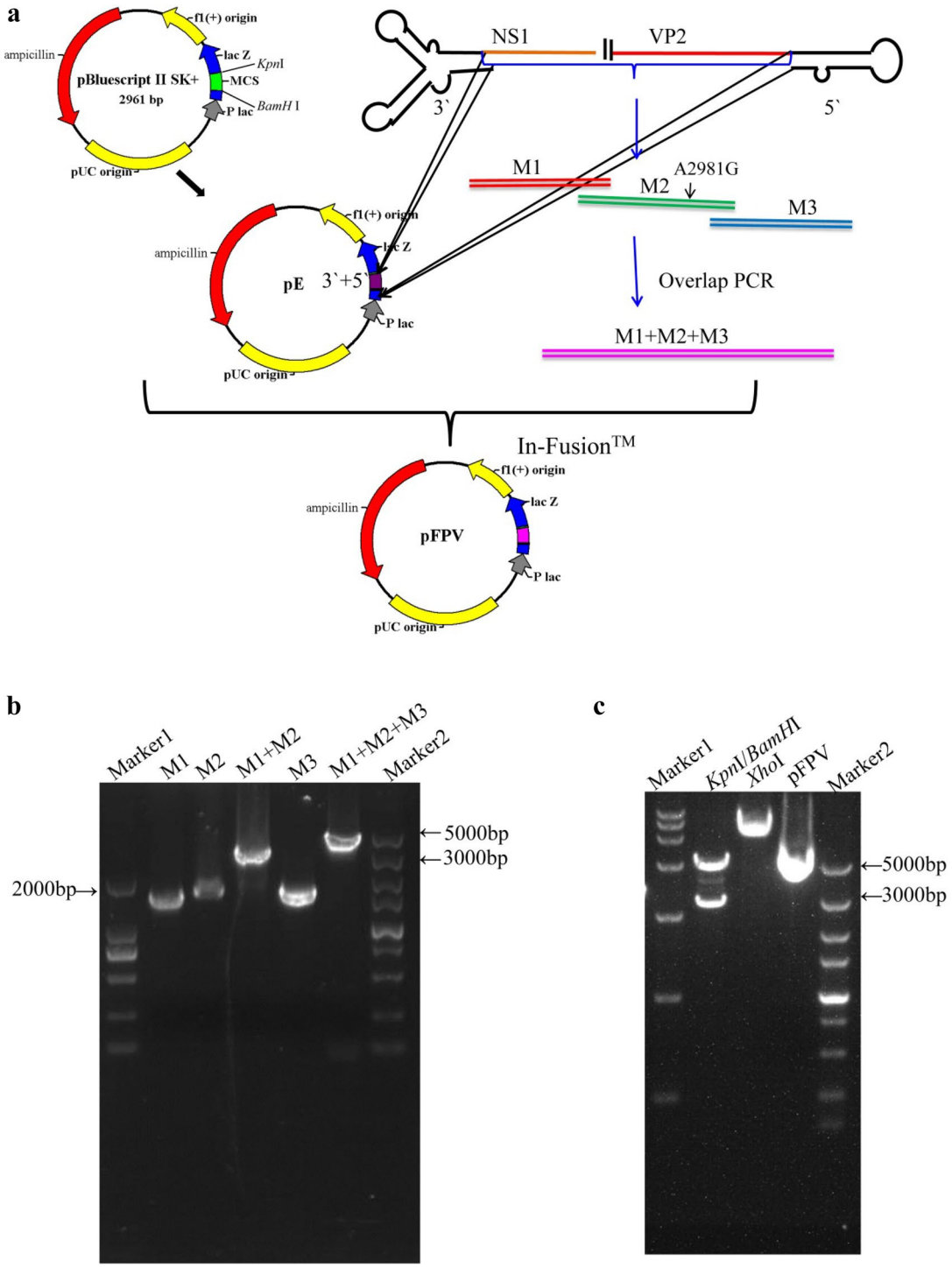

Fig. 1 **a** Molecular cloning of full-length FPV DNA (pFPV). The 3′ and 5′ hairpin regions of the genome were inserted into the pBluescript II SK (+) vector with restriction sites *Kpn*I and *Bam*HI to form the pE vector. Three fragments (M1, M2 and M3) covering the FPV middle region were inserted into the pE vector to obtain the full-length cDNA clone of FPV, named pFPV. A silent mutation was introduced at 2981 bp, which formed a unique *Xho*I restriction enzyme site. **b** PCR results of the middle region: M1, M2, M1 + M2, M3, and M1 + M2 + M3 fragments. **c** Enzyme digestion of plasmids pE by *Kpn*I/*Bam*HI and *Xho*I

laser scanning microscopy in HH-1/86 or $rFPV_5$, but not in the uninfected cells (Fig. 3a). Transient expression of the VP2 protein after transfection of F81 cells was assessed by an indirect immunofluorescence assay. Tested for HA, the the titer of the rFPV had reached the level of the parental FPV strain HH-1/86, 2^{11}, by the 10th passage (Fig. 3b). Cell

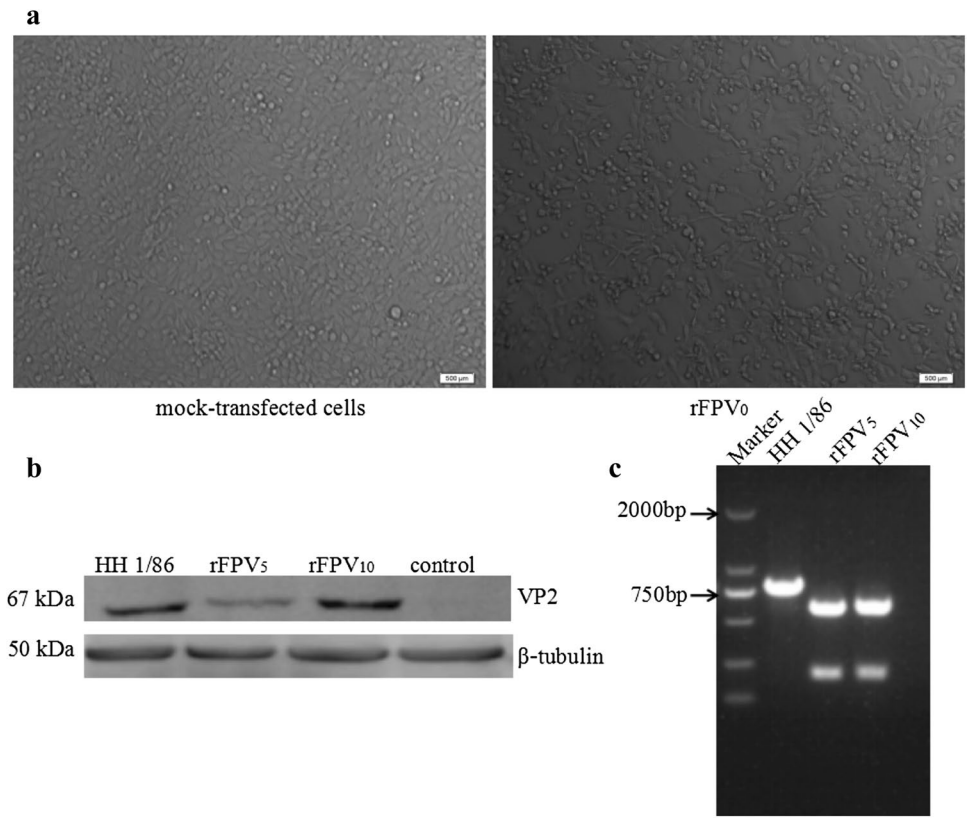

Fig. 2 **a** F81 cells were infected with $rFPV_0$ was successfully used to infect F81 cells, with typical FPV CPE developing by day 3 post-infection. While mock-transfected cells remained normal. **b** VP2 expression in rFPV-infected F81 cells by Western blotting with uninfected cells as negative control. **c** PCR products of viral DNA from HH-1/86, $rFPV_5$ and $rFPV_{10}$ cultures and treated with *Xho*I

culture media from $rFPV_5$ and $rFPV_{10}$ were collected every 12 h after infection and tested by $TCID_{50}$ assay. Results were identical to that of parent virus HH-1/86 and the rescued FPV had similar growth kinetics to that of the parent strain (Fig. 3c).

Discussion

The HH-1/86 strain is 100% and 99.8% similar to FPV strains MG132167A (GenBank accession number: KP769859) and XJ-1 (GenBank accession number: EF988660), respectively [25, 26]. There have been several reports of infectious clones of parvoviruses. Parrish et al. [27, 28] proposed that residues 93, 103, or 323 in the VP2 protein determine the host range and antigenic characteristics of CPV and hypothesized that these amino acid sites may be associated with antigenic determination and HA features [29]. The [93 (N) 103 (A) 323 (D)] amino acids sites are conserved in the HH-1/86.

There are several difficulties associated with the construction of a full-length infectious clone of FPV. First, the sequences of the two ends of the virus are hard to obtain by PCR, which was also the reason why complete genomes of parvoviruses have seldom been reported. Second, the secondary structures of the ITRs must be preserved because these are important for viral replication. It is impossible to construct an infectious clone if only primary sequences are used [30].

Although the sequences of the ITRs of the virus can be obtained by pre-degeneration PCR, the palindromic repeats, which contain secondary structures, are hard to obtain by cloning in vitro. Thus, the structures must be generated artificially. In-fusion technology can connect synthetic ITR sequences and the linear sequence obtained by PCR and can generate clones with high rates of expression. We generated a silent mutation in the sequence of the VP2 protein and formed an *Xho*I restriction enzyme recognition site in the infectious clone. As a result, the rescued virus could be easily identified, and the features of the rescued virus remained very similar to those of the parent virus [31]. Kariatsumari et al. [32] obtained the whole-genome sequence of MEV and constructed an infectious clone of it. In 2014, Yuan et al. [31] constructed an infectious clone of MEV by In-fusion technology, but the sequences of the ITRs referenced the MEV strain Abashiri since the whole genome was not sequenced. Han et al. [33] completed the full-length genomic characterization by partial degeneration before PCR.

In the present study, the hairpin structures on both ends were artificial and were cloned into the pBluescript II SK (+) vector, whereas the middle region of the virus was obtained by overlap PCR, joining linearized pE by In-Fusion™ HD enzyme premix to form the full-length infectious clone pFPV. The pFPV clone was then used

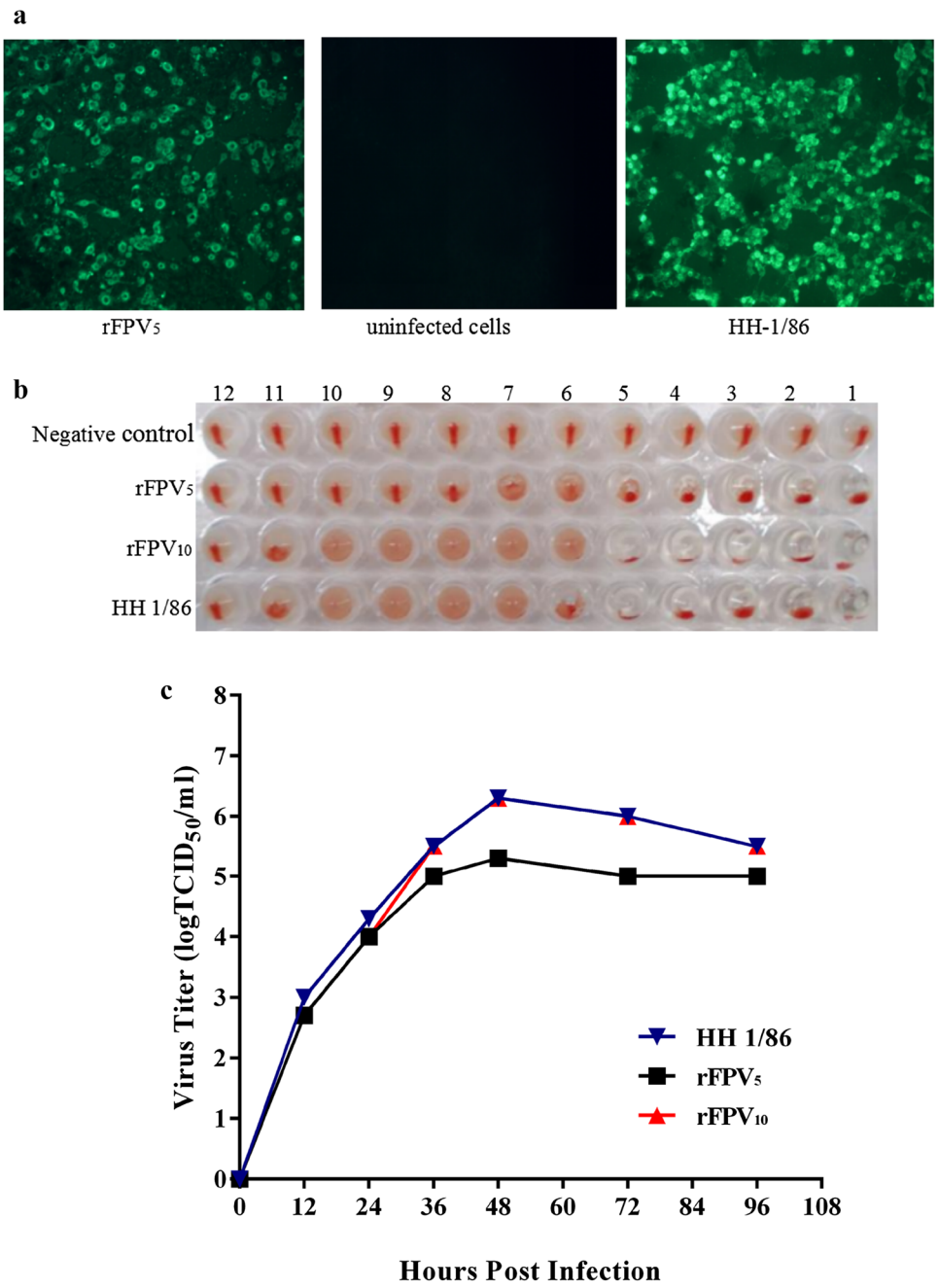

Fig. 3 **a** F81 cells were obtained 48 h after transfection and incubated with mouse-anti-VP2 and FITC-rabbit-anti-mouse IgG antibodies. **b** HA titers of FPV strains HH-1/86, $rFPV_5$ and $rFPV_{10}$. **c** F81 cells were infected with FPV HH-1/86, $rFPV_5$, or $rFPV_{10}$ at an MOI of 0.1, and culture media collected between 0 and 96 h for titration

to transfect F81 cells, and a rescued virus (rFPV) was obtained. The rescued virus was identified by CPE, hemagglutination, protein expression, viral growth, and genetic marker assays and was indistinguishable from the parent virus.

The present study describes an opportunity to create mutated viruses for studying the structures and functions of FPV proteins and the hairpins on both ends in viral replication and infection [34]. An infectious clone will also help in the exploration of the pathogenesis of FPV [35].

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Author contributions SY, YZ and WL conceived and designed the experiments. NC and WZ performed the experiments. JX, YY, HW, YG, and XX analyzed the data. NC and WZ wrote the article. GL managed and submitted the paper.

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Compliance with ethical standards

Conflict of interest The authors do not have any conflicts of interest.

Ethical approval This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals developed by the China Council on Animal Care. All protocols were approved by the Committee on the Ethics of Animal Experiments of the Institute of Military Veterinary at the Academy of Military Medical Sciences. The approved ID or permit numbers were SCXK-2012-017.

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