Virology Reports 6 (2016) 97–103

Contents lists available at ScienceDirect

Virology Reports

journal homepage: www.elsevier.com/locate/virep

Rescue of duck-origin virulent Newcastle disease virus from cloned cDNA and stable expression of the red fluorescent protein

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ARTICLE INFO

Article history:
Received 1 August 2016
Received in revised form 19 October 2016
Accepted 19 October 2016
Available online 20 October 2016

Keywords:
Newcastle disease virus
Reverse genetics
Genotype VII
Red fluorescent protein

ABSTRACT

Ducks are generally considered as potential reservoirs for different genotypes of Newcastle disease virus (NDV) and to be resistant even to velogenic NDV strains. However, outbreaks of highly virulent genotype VII NDV lethal to ducks have been frequently reported in China in recent years. But until now, the pathogenesis and potential vaccine of duck-origin genotype VII NDV are not known. In this study, a reverse genetics system using the prevalent high virulence genotype VIId isolate SS1 was constructed. Based on this system, a red fluorescent protein (RFP)-expressing virus was generated by inserting an additional transcription cassette coding for the RFP between the noncoding region of P and M genes. The rescue of the recombinant viruses was confirmed by western blotting, fluorescence microscopy and genetic marker detection. In addition, the replication kinetics, biological characteristics and pathogenicity of the rescued viruses were indistinguishable from the parental wild-type virus. Moreover, the recombinant virus rSS1-RFP could efficiently replicate in most of the duck tissues, especially in duck immune organs. The results obtained suggest that this reverse genetics system will provide a useful tool for the analysis of duck-origin NDV pathogenesis and dissemination, as well as preparation for novel vaccine vector or genotype-matched NDV attenuated vaccines used in ducks.

Newcastle disease virus (NDV), also known as avian paramyxovirus serotype-1 (APMV-1), is a member of the genus *Avulavirus* within the *Paramyxoviridae* family (Gogoi et al., 2015). The genome of NDV is a negative-sense, non-segmented, single-stranded RNA that contains six major genes in the order of 3′-NP-P-M-F-HN-L-5′ and encodes six structural proteins as well as two non-structural proteins, V and W (Miller et al., 2010; Yusoff and Tan, 2001). Up to now, more than 250 bird species are reported to be susceptible to NDV, thus causing severe economic burden in domestic poultry industry worldwide (Ganar et al., 2014). In general, ducks are considered as the natural reservoir for all genotypes of NDV and are typically experienced asymptomatic infection or little pathology for velogenic NDV strains lethal to chickens (Liu et al., 2008). However, outbreaks of high virulence genotype

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http://dx.doi.org/10.1016/j.virep.2016.10.002

VII NDV strain lethal to ducks have been occasionally reported in China in recent years (Dai et al., 2014; Kang et al., 2014; Kang et al., 2016), but the pathogenesis of genotype VII NDV in ducks has not been clearly clarified. Therefore, it is worth strengthening fundamental research on the genetic evolution and pathogenic mechanism of virulent NDV isolates.

Nowadays, the development of plasmid-based reverse genetics systems to rescue negative-sense RNA viruses from cloned cDNA provides a means not only to investigate the pathogenesis of virus (Kai et al., 2015; Zhao et al., 2013), but also to make it possible to generate novel vaccines and vaccine vectors (Neumann et al., 2002; Duan et al., 2015a). Since the generation of infectious NDV was firstly reported in 1999, the recovery of other NDV strains, such as Herts/33 (de Leeuw et al., 2005), ZJ1 (Liu et al., 2007), I4 (Hu et al., 2011), 9a5b-D5C1 (Yu et al., 2012) and NA-1 (Wang et al., 2015), was also successfully accomplished. It is noteworthy that all of the rescued NDV strains were isolated from chicken or goose flocks, the recovery of duck-origin virulent NDV strain has not been reported. In this study, we used the established reverse genetics system based on the full-length cDNA under the control of the T7 promoter to rescue duck-origin genotype VII NDV strain SS1. Furthermore, the recombinant virus rSS1-RFP expressing the red fluorescent protein (RFP) was produced. The stability and pathogenicity of rSS1-RFP was also evaluated.

The SS1 strain that caused about 30% mortality was isolated from Sansui Sheldrake duck flocks in Guizhou Province, in 2014 (Duan et al., 2015b). The complete genomic sequence of the isolate has been determined and deposited in the GenBank database (accession no. KP742770). To construct the three helper plasmids, the open reading frames (ORFs) of the NP, P and L genes were amplified from the SS1 cDNA using specific primers (Supplemental Table S1), and then cloned into pCI-neo vector (Promega, USA) to generate pCI-NP, pCI-P, and pCI-L, respectively. To assemble the full-length SS1 cDNA into the TVT7R(0.0) vector, seven overlapping PCR fragments spanning the full-length cDNA of SS1 were generated by RT-PCR with specific primers (Table 1). To conveniently insert foreign genes into SS1 genome as previously reported (Hu et al., 2007; Zhao et al., 2015), the F3 fragment containing the *Apa*I restriction site at the 4118–4123 nt in the M ORF region was eliminated by introducing one synonymous mutation at position 4120 (G to A), and served as the genetic marker, while another *Apa*I restriction site at the 3139–3144 nt in the P-M noncoding region was reserved as an insertion locus for the foreign genes (Fig. 1A). The obtained seven fragments were subsequently ligated into the TVT7R(0.0) vector to generate the full-length cDNA cloning plasmid pTVT/SS1 by utilizing unique restriction enzyme sites (Fig. 1A). For the construction of pTVT/SS1-RFP, the “gene end” (GE), intergenic (IG), “gene start” (GS) and the *Apa*I restriction sites sequences (Hu et al., 2007) were added to the RFP ORF by PCR amplification using primers RFP-F and RFP-R (Table 1), according to the “rule of six” (Fig. 1B). The amplicon was inserted into the F3 fragment, which was then digested with *Age*I and *Bst*Z17I to replace the corresponding region in the full-length cDNA clone pTVT/SS1 (Fig. 1B). The resulting plasmid was named pTVT/SS1-RFP.

To rescue infectious virus from the plasmids, BSR-T7/5 cells stably expressing the T7 phage RNA polymerase were grown to 80% confluence in 35-mm-diameter dishes and then cotransfected with a total of 3 μg DNA consisting of a mixture of pTVT/SS1 or pTVT/SS1-RFP together with three helper plasmids at a ratio of 2:2:1:1 using the FuGENE HD transfection reagent (Roche, Germany). At 60 h post-transfection, the cell monolayers and culture supernatants were harvested and inoculated into the allantoic cavities of 10-day-old embryonated SPF eggs. The hemagglutination (HA) test revealed that HA-positive allantoic

Table 1
Primers used for the construction of full-length cDNA and helper plasmids.

Primer name	Position	Nucleotide sequence (5′ → 3′)	Length (bp)
P1F	1–22	***CGTCTCGTATA***GGGACCAAACAGAGAATCTGTGAGG (*Bsm*BI)	1803
P1R	1779–1803	TTCTAATACCTTGGGCTCTGGGCGG	
P2F	1738–1763	CCACCCGGGACAACACAGGCACAGC	1204
P2R	2911–2941	ACG***ACGCGT***TCAGGCCCGCTTGCAGTGGCAGAC (*Mlu*I)	
P3F	2859–2884	ACGCTCAATAAACTCTCACAACCGG	1876
P3R	4710–4734	ATGATTGACCCAGTCTGAGACGAGG	
P4F	4661–4689	GCAGCTGCAGGAATTGTAGTAACAGGAG	2713
P4R	7348–7373	CCTCCGTAAACTGGGAACCATACACG	
P5F	7318–7346	GGAGTGGGAGGAGGGTCTTTTATTGACG	4704
P5R	11,997–12,021	GACATGTGGGCTATTTTCGCGAGCG	
P6F	11,969–11,994	TCGAAGACTCAAGAGAGGAGAGCCG	1126
P6R	13,070–13,094	ACAGCAGATCGTACTTATTCCCTGC	
P7F	13,028–13,053	TTGTTAGTCTCTTGCACTCGACGCG	2165
P7R	15,168–15,192	***CGTCTCTA***CCCACCAAACAGAGATTTGGTGAATGAC (*Bsm*BI)	
NP-F	78–99	CG***GAATTC***GAGCGCGAGGCCGAAGCTTGAAC (*Eco*RI)	1544
NP-R	1585–1606	AT***GTCGAC***CTGGGTGTTGTCGATCAGTAC (*Sal*I)	
P-F	1877–1901	CCG***CTCGAG***GTGAATTAGGGTGAAGATGGCCACT (*Xho*I)	1256
P-L	3092–3144	GCC***GTCGAC***AGTGACGGGAGCCTGTTGTGAGT (*Sal*I)	
L-F1	8385–8412	ACG***GTCGAC***ACATGGCGGGCTCCGGTCCAGAAAGGGC (*Sal*I)	4696
L-R1	13,041–13,071	GCATATAAACCTGAGACC***ACGCGT***CGAGTGC (*Mlu*I)	
L-F2	13,034–13,059	GTCTCTTGCACTCG***ACGCGT***GGTCTC (*Mlu*I)	1980
L-R2	14,983–15,001	ATAT***GCGGCCGC***TTAAGAGTCATTATTACTG (*Not*I)	
RFP-F		CT***GGGCCC***TC*ttagaaaaaa*TacgggtagaaGTACCATGGCCTCCTCCG AGAACGTC (*Apa*I)	
RFP-R		TT***GGGCCC***TCTTACAGGAACAGGTGGTGGCGGCCCTCGGTG CGCTCG (*Apa*I)	

The restriction enzyme sites used in the primers are marked in italic and bold type. The gene end sequence is lowercased and italicized. The gene start sequence is lowercased.

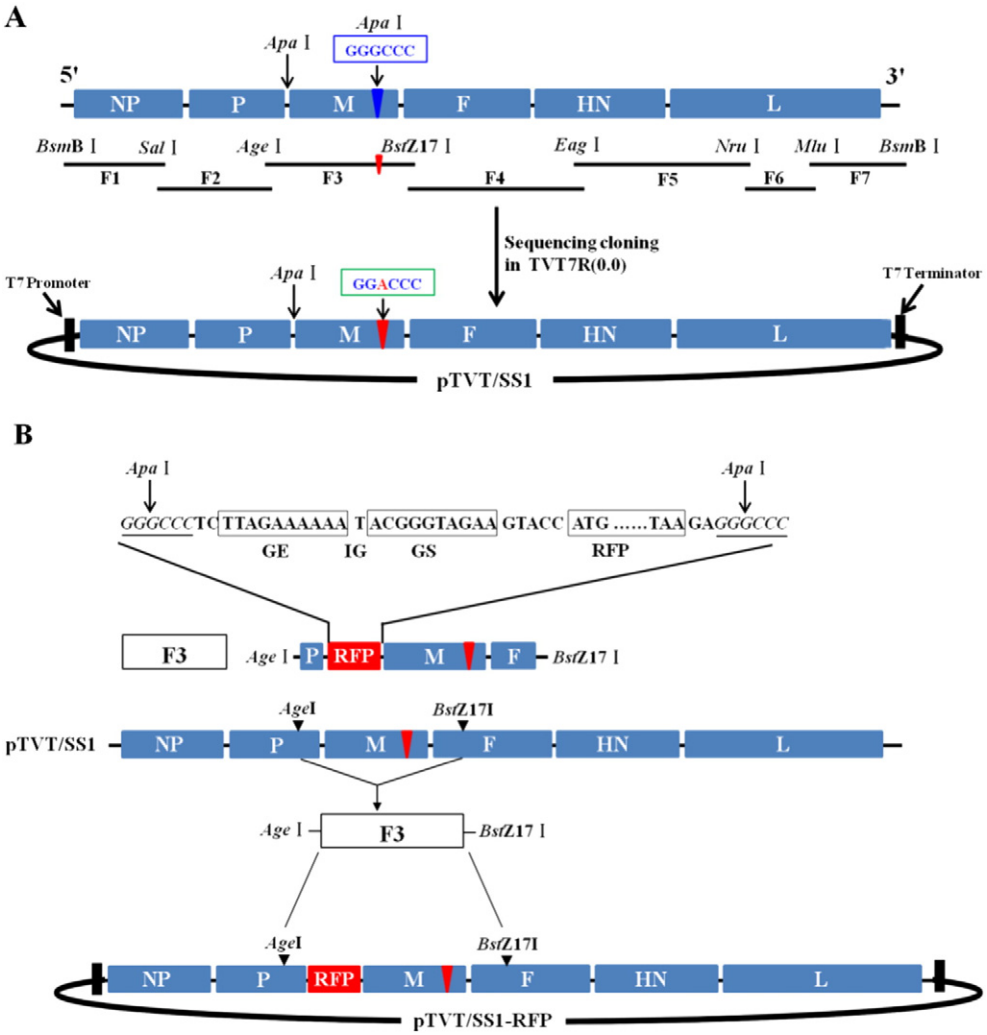

Fig. 1. Schematic representation of the construction of full-length cDNA clone of pTVT/SS1 and pTVT/SS1-RFP. (A) Seven overlapping PCR fragments spanning the full-length cDNA of SS1 were generated. The F3 fragment containing the *Apa*I restriction site at the 4118–4123 nt in the M ORF region was eliminated by introducing one synonymous mutation at position 4120 (G to A), and served as the genetic marker. The fragments were then ligated into the TVT7R(0.0) vector to generate the full-length cDNA cloning plasmid pTVT/SS1. (B) An artificial transcription cassette containing the GE, IG, GS and the *Apa*I restriction sites sequences were added to the RFP ORF and then inserted into the F3 fragment. The new obtained fragment was digested with *Age*I and *Bst*Z17I to replace the corresponding region in the pTVT/SS1 to generate pTVT/SS1-RFP.

fluids were detected in the rescued viruses rSS1 and rSS1-RFP. To unambiguously verify that the rescued viruses were derived from the infectious clone rather than wild-type NDV contamination, the presence of artificially introduced genetic marker in five times serially passaged rSS1 or rSS1-RFP was confirmed by DNA sequencing (Invitrogen, Shanghai, China) (Fig. 2A). BSR-T7/5 cells were subsequently infected with the viruses at a multiplicity of infection (MOI) of 0.1 and then detected by fluorescent assay using chicken NDV antiserum at 12 h post-infection (hpi). As shown in Fig. 2B, green fluorescence were detected in rSS1 and rSS1-RFP infected cells, but not in mock-infected cells. Meanwhile, red fluorescence could be directly observed in rSS1-RFP infected cells (Fig. 2B). On the other hand, the lysate supernatants of virus infected cells were collected to verify the normal expression of viral protein as well as the foreign RFP at 48 hpi. As expected, NDV M protein was detected by rabbit anti-M polyclonal antibody in both rSS1 and rSS1-RFP infected cells, but the RFP was only detected in rSS1-RFP infected cells, whereas no bands were observed in mock-infected cells (Fig. 2C). These results convincingly indicated the successful rescue of rSS1 and rSS1-RFP from the infectious cDNA clone.

Next, we evaluated the replication kinetics of the rescued viruses. When BSR-T7/5 cells were infected with the viruses at an MOI of 0.1, the RFP was easily observed in live cells using fluorescence microscope and was strong enough at 24 hpi accompanied by slight cytopathic effect (CPE). Almost all of the cells exhibited CPE at 48 hpi and cell monolayers were completely destroyed at 60 hpi. No fluorescence signal was detected in rSS1 or mock-infected cells (Fig. 3A). To further compare the replication capacity of the parental and recombinant viruses, plaque assay was performed as previously described (Duan et al., 2014). The plaques produced by the viruses were measured using the GNU image manipulation program version 2.8 (Negovetich and Webster, 2010). As

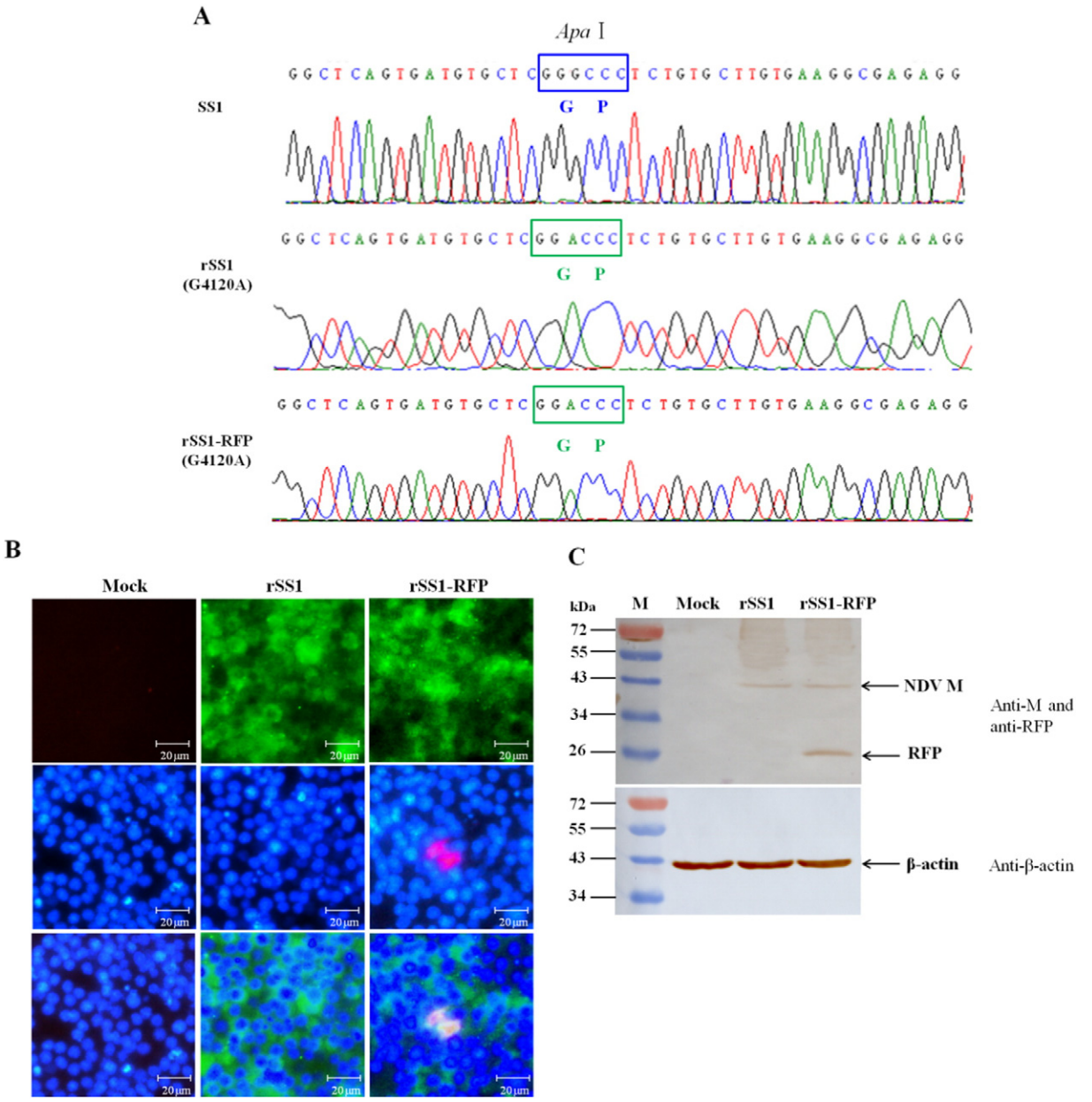

Fig. 2. Characterization of the rescued NDV viruses. (A) The presence of artificially introduced genetic marker (G4120A) in five times serially passaged rSS1 and rSS1-RFP was confirmed by DNA sequencing. (B) BSR-T7/5 cells were infected with the rescued viruses at an MOI of 0.1 and then detected by fluorescent assay using chicken NDV antiserum at 12 hpi. DAPI was used to stain nuclei (×200 magnification). Representative images are shown. Scale bar, 20 μm. (C) BSR-T7/5 cells were infected with rSS1 or rSS1-RFP at an MOI of 0.1. The lysate supernatants of cells were collected to verify the expression of NDV M protein and RFP at 48 hpi using rabbit anti-M polyclonal antibody, mouse anti-RFP monoclonal antibody, or mouse anti-β-actin monoclonal antibody. Binding was visualized with 3,3-diaminobenzidine reagent after incubation with horseradish peroxidase-conjugated secondary antibodies.

shown in Fig. 3B, DF-1 cells infected with rSS1 or rSS1-RFP developed the similar plaques as SS1, with a mean size of 2.58 ± 0.36 mm, 2.55 ± 0.43 mm and 2.61 ± 0.36 mm, respectively. In addition, the results of *in vitro* growth characteristics of these viruses showed that there was no discernible difference in the growth kinetics or the maximum virus titers among the viruses at any given time point (Fig. 3C).

To compare the biological characteristics of the parental and recovered viruses, the mean death time (MDT), the intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index (IVPI) were measured according to the standard procedures (Alexander, 2000). The results showed that the MDT of rSS1 and rSS1-RFP was 54.2 and 58 h, respectively, and the ICPI and IVPI values were 1.90 and 1.88, 2.62 and 2.74, respectively, which were similar to those of the parental virus (Table 2). The EID_{50} and $TCID_{50}$ of rSS1 and rSS1-RFP were $10^{8.33}$ and $10^{8.67}$, $10^{8.17}$ and $10^{8.25}$, respectively, which showed no significant

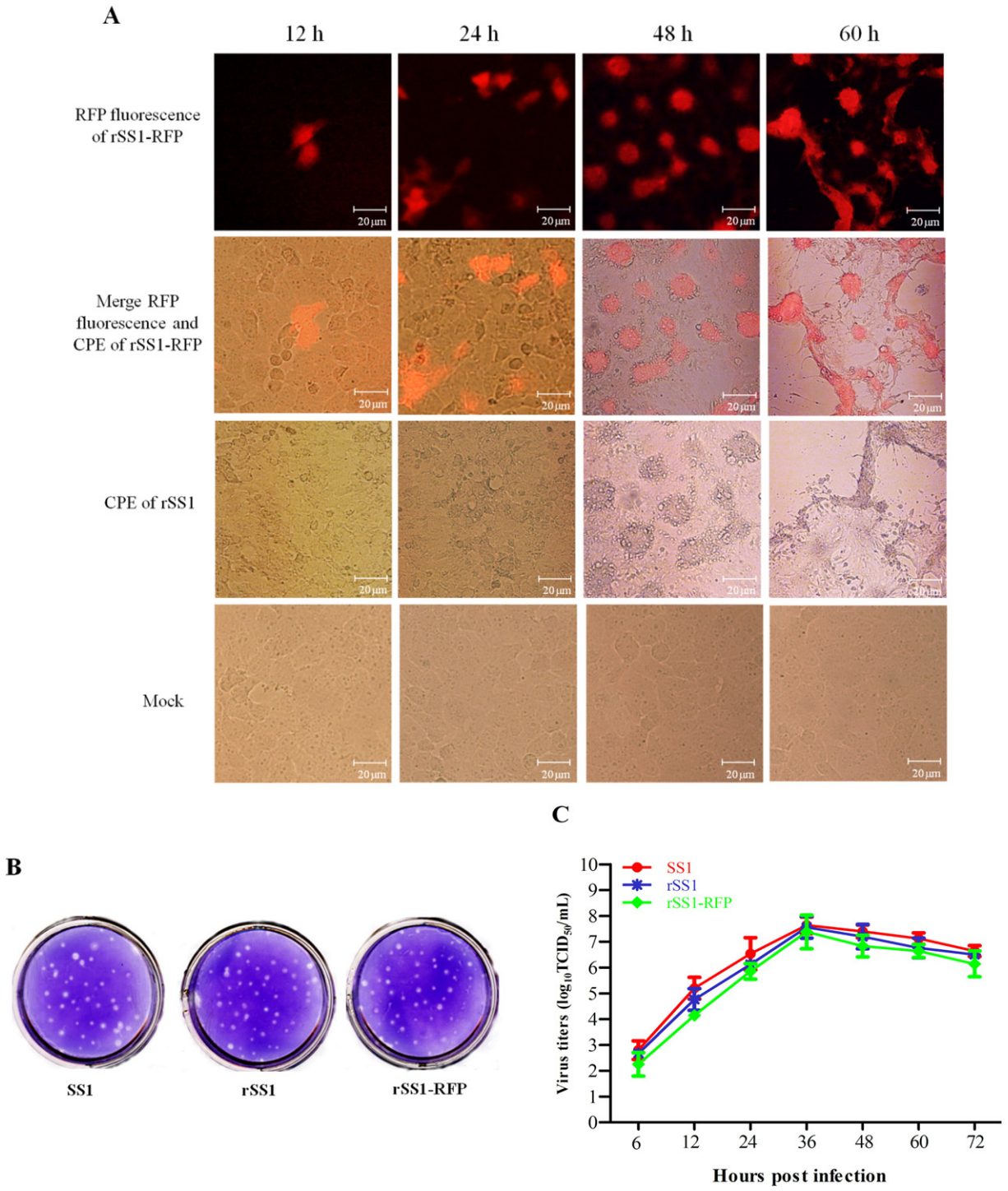

Fig. 3. The replication kinetics of rSS1 and rSS1-RFP in infected cells. (A) Analysis of the replication capacity of the rescued viruses by cytopathic effect and the RFP fluorescence. BSR-T7/5 cells were infected with rSS1 or rSS1-RFP at an MOI of 0.1. The infected cells were observed at different time points under the inverted fluorescence microscope and optical microscope (×200 magnification). Scale bar, 20 μm. (B) Plaque assay of the parental and rescued viruses in DF-1 cells. After 48 h of incubation at 37 °C, the agarose was removed and the cells were fixed with 10% formaldehyde and then stained with 0.1% crystal violet. The sizes of plaques formed by the viruses in DF-1 cells are shown. The average plaque size of 10 representative plaques for each virus is indicated. (C) Growth kinetics curves of the parental and rescued viruses in DF-1 cells. Cells were infected with the viruses at an MOI of 0.1. Viruses were harvested at 12 h intervals, and virus titers were determined in DF-1 cells and expressed as $TCID_{50}$/mL. Values represent the mean and SD of three independent tests.

Table 2
Biological characteristics of the parental and recovered viruses.

Virus	Pathogenicity			Virus titer		
	MDT (h)	ICPI	IVPI	EID_{50}/mL	$TCID_{50}$/mL	HA
SS1	52	1.89	2.80	$10^{8.83}$	$10^{8.50}$	8 log_2
rSS1	54.2	1.90	2.62	$10^{8.33}$	$10^{8.17}$	8 log_2
rSS1-RFP	58	1.88	2.74	$10^{8.67}$	$10^{8.25}$	7 log_2

difference when compared to the parental virus (Table 2). In addition, the *in vivo* pathogenicity of the rescued viruses in ducks was also evaluated. 20 ten-day-old Sansui Sheldrake ducks were randomly allocated in four experimental groups, consisting of SS1 (n = 5), rSS1 (n = 5), rSS1-RFP (n = 5) and mock (PBS, n = 5) infected groups. Ducks were inoculated intranasally with $10^{6.0}$ EID_{50} of each virus in a volume of 0.1 mL. The results showed that ducks inoculated with the virus rSS1 or rSS1-RFP presented the similar typical clinical symptoms and gross lesions as birds infected with SS1, whereas birds inoculated with PBS presented no clinical signs (Supplemental Table S1). Moreover, red fluorescence was obviously observed in the tissue smears of trachea, spleen, kidney, duodenum and bursa of fabricius in rSS1-RFP infected ducks at 4 days post-infection (Fig. 4A). Furthermore, strong positive antigen staining was also presented in the above tissues (Fig. 4B), indicating that SS1 strain could efficiently replicate in duck tissues and might be used as candidate vaccine strain against Newcastle disease in ducks. Taken together, these data demonstrated that the growth characteristics, biological properties and pathogenicity of the rescued viruses are similar to those of the parental wild-type virus.

In conclusion, we have successfully rescued a highly virulent duck-origin genotype VII NDV strain from cloned cDNA. Based on this reverse genetics system, the foreign RFP gene could be efficiently expressed in rSS1-RFP infected cells or parts of duck tissues, and did not affect the growth characteristics and pathogenicity of the rescued virus. These results undoubtedly indicate that this system will make it possible to strengthen the fundamental research of duck-origin NDV pathogenesis and dissemination, as well as to generate novel vaccine vector or genotype-matched NDV attenuated vaccines for ducks.

Supplementary data to this article can be found online at doi:10.1016/j.virep.2016.10.002.

Conflict of interest

The authors declare no conflict of interest.

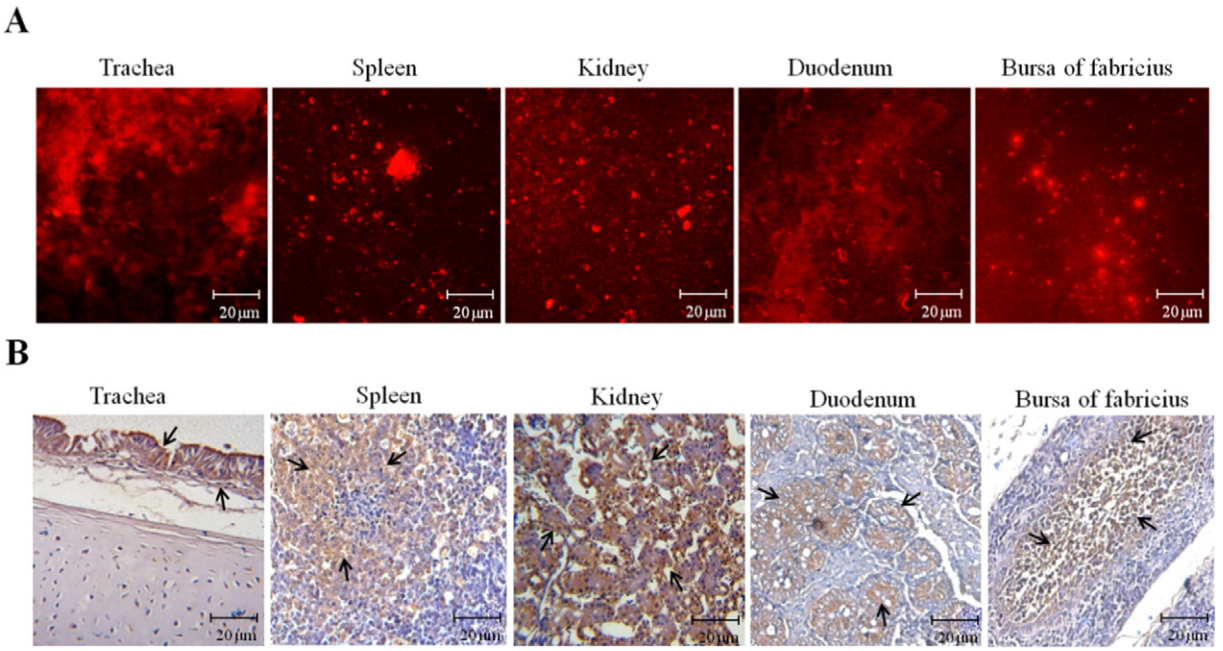

Fig. 4. Detection of RFP fluorescence and NDV antigen in the tissues of rSS1-RFP infected ducks. (A) 10-day-old Sansui Sheldrake ducks were inoculated intranasally with $10^{6.0}$ EID_{50} of rSS1-RFP in a volume of 0.1 mL. The RFP fluorescence in the tissue smears of trachea, spleen, kidney, duodenum and bursa of fabricius was observed under the inverted fluorescence microscope at 4 days post-infection (×200 magnification). Representative images are shown. Scale bar, 20 μm. (B) Detection of NDV antigen in rSS1-RFP infected ducks at 4 days post-infection. Photomicrographs illustrated immunohistochemistry on sections of trachea, spleen, kidney, duodenum and bursa of fabricius. The immunohistochemical detection of HN protein is indicated with arrows (×200 magnification). Scale bar, 20 μm.

Acknowledgments

We thank for Dr. Andrew Ball (University of Alabama, USA) for providing the transcription vector TVT7R(0.0). This work was supported by the Scientific Research Cooperation Project with America and Oceania Region, Ministry of Education (JWSM-2014-2029), the Science and Technology Fund of Guizhou Province (QKH-2015-2054), the Guizhou Province-School Cooperation Project (QKH-LH-2014-7669), the Chinese Special Fund for Agro-scientific Research in the Public Interest (201303033) and the Scientific Research Project of Guizhou University Talents Fund (GDRJHZ-2014-10).

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