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Foreign gene expression attenuates a virulent Newcastle disease virus in chickens

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Abstract

Newcastle disease virus (NDV) is an important pathogen for poultry and is used as a vector for developing novel poultry vaccines. Previous studies showed that foreign gene insertion in NDV vector decreases virulence determined by in vitro assays; however, the impact of foreign gene expression on the pathogenicity of NDV in susceptible chickens is not fully investigated. In this study, a recombinant NDV based on a velogenic strain carrying the orange fluorescent protein (OFP) gene between the phosphoprotein (P) and matrix (M) genes was generated using reverse genetics. Biological characteristics, including virus replication, virulence, and OFP expression, and the pathogenicity in chickens were evaluated. The recombinant NDV showed comparable replication capacity in eggs and cells as the parental virus, whereas OFP insertion resulted in a mild impairment of virulence, evidenced by longer mean death time in embryos. High OFP expression was detected in the cells inoculated with the recombinant NDV. In addition, the recombinant NDV induced delayed onset of disease, lower severity of clinical signs, and lower mortality in chickens compared to the parental virus. Moreover, high titers of the parental virus were detected in the spleen, lung, and intestinal tract, while no recombinant NDV was recovered from these tissues. Our findings suggest that in vitro characteristics related to the insertion of the OFP gene in a virulent NDV do not correlate to alteration of the pathogenicity in chickens. Our results provided new information regarding assessment of the impact of foreign gene expression on the pathogenicity of NDV.

Keywords Newcastle disease virus · Foreign gene expression · Orange fluorescent protein · Reverse genetics · Pathogenicity · Virus vector

Introduction

Newcastle disease (ND) is an acute and highly contagious infectious disease caused by Newcastle disease virus (NDV), which affects different poultry species and seriously threatens the development of poultry industry. NDV is a member of the genus *Orthoavulavirus* in the family of *Paramyxoviridae* [1]. The genome of NDV is a non-segmented, single-stranded, negative-sense RNA that contains six genes encoding the nucleocapsid protein (NP), matrix protein (M), phosphoprotein (P), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (L) [2]. Two additional proteins, V and W, are produced by RNA editing of the P gene [2]. NDV strains can be categorized into three main pathotypes: velogenic, mesogenic, and lentogenic, depending on the severity of the disease they induce. With the establishment of reverse genetics, multiple molecular determinants for the virulence of NDV were

identified, and the cleavage site of the F protein is identified as the major determinant [3]. In addition, NDV is used as a vector to express protective antigens to develop new poultry vaccines and cytokines for viral pathogenesis studies [4–7].

The insertion site of foreign genes in the NDV genome exerts a critical influence on virus replication and foreign gene expression. An early study reported that insertion of the chloramphenicol acetyltransferase (CAT) gene immediately before the open reading frame (ORF) of the 3′-proximal NP gene of La Sota strain resulted in high CAT expression and marginal change in pathogenesis [8]. Subsequently, different foreign genes were inserted into multiple regions in the NDV genome, and the P-M gene junction was identified as the optimal insertion site for foreign genes [9]. Similar findings were also observed in avian paramyxovirus serotype 3, a type of virus closely related to NDV [10]. Notably, the majority of these studies were performed in lentogenic NDV backbones, and the effect of foreign gene insertion was mainly assessed by measuring in vitro virus replication and foreign gene expression level.

However, studies elucidating the impact of foreign gene insertion in mesogenic or velogenic NDV backbones were limited. The first report of foreign gene expression in NDV vector revealed that insertion of the CAT gene between the HN and L genes of mesogenic BC strain resulted in apparent growth retardation and attenuation in virulence [11]. A recombinant velogenic NDV expressing the GFP reporter (rZJ1-GFP) caused severe clinical signs and pathological lesions in 10-day-old chickens, indicating its high pathogenicity in chickens [12]. Pathogenicity of rZJ1-GFP was also tested in other independent experiments, which consistently suggested that the virus had slightly decreased intracerebral pathogenicity index (ICPI) values compared to the parental virus [6]. Nevertheless, different pathogenicity profiles of rZJ1-GFP were observed in 4-week-old chickens. Susta et al. showed that rZJ1-GFP caused severe neurological signs at day 5 post infection, and thus, all chickens were recorded as dead [13]. In contrast, Marcano et al. detected apparent attenuation of this virus in chickens, with only 20% mortality at day 5 and survival of two chickens at day 10 after infection [6]. Additionally, expressing of different chicken cytokines, such as interferon-γ (IFN-γ), interleukin (IL)-2 or IL-4, in ZJ1 caused attenuation of the pathogenicity, decrease in virus load or changes in pathological manifestation in chickens. These effects may be mainly associated with the antiviral activity or immunomodulatory function of the cytokines expressed in NDV [6, 7, 13]. Therefore, the impact of foreign gene insertion on the pathogenicity of NDV in susceptible chickens is an open question.

In this study, we generated a recombinant virus expressing the orange fluorescent protein (OFP) gene between the P and M genes in a velogenic NDV using reverse genetics. In vitro biological characteristics and the pathogenicity of the recombinant virus in chickens were investigated. The recombinant virus had comparable replication in cells and mean death time (MDT) values in chicken embryos as the parental virus, whereas it showed significantly decreased pathogenicity and virus replication in chickens. Our findings suggest that the OFP gene insertion between the P and M genes in a virulent NDV results in no substantial changes of in vitro features but significant virus attenuation in chickens.

Materials and methods

Ethics statements

All experiments involving virulent NDV strains were performed in animal biosafety level-3 facilities.

Cells, viruses and plasmids

Chicken embryo fibroblasts (CEFs) were grown in M199 medium containing 4% fetal calf serum (FCS) and penicillin, streptomycin, and amphotericin. DF1 and BHK-21 cells (clone BSR-T7/5) that express T7 RNA polymerase were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FCS, and BHK-21 cells were used for virus rescue. A reverse genetics-derived genotype VII NDV strain JS5/05 [14] was used as the parental virus. The virus was propagated in 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECEs). The full-length (FL) cDNA clone of JS5/05 and three helper plasmids encoding the NP, P, and L proteins were constructed previously [15]. The plasmid pCMV-C-mOrange2 (Beyotime, Nantong, China) was used as the template to amplify the OFP gene.

Construction of the recombinant NDV cDNA clone containing the OFP gene

The OFP gene was inserted into the genome of JS5/05 using a combined cloning strategy of in-fusion and ligation. Firstly, the pCMV-C-mOrange2 plasmid was used as a template to amplify the OFP gene using the primers (OFP-F and OFP-R). The NDV transcriptional signals, including the gene end, intergenic, and gene start sequences, were added to the upstream of the OFP gene using the forward primer OFP-F. As illustrated in Fig. 1, two gene segments of JS5/05, designated as JS5/05-P and JS5/05-MF, were PCR-amplified using the primer sets (JS5/05-P-F/JS5/05-P-R and JS5/05-MF-F/JS5/05-MF-R), respectively. Then, these fragments were cloned into the linearized pCR2.1 TOPO vector using a Ready-to-Use Seamless Cloning Kit (Sangon, Shanghai, China). After identification by restriction endonuclease digestion and sequencing, the combined gene fragment was transferred to the FL plasmid pNDV/JS5/05 using *Age*I and
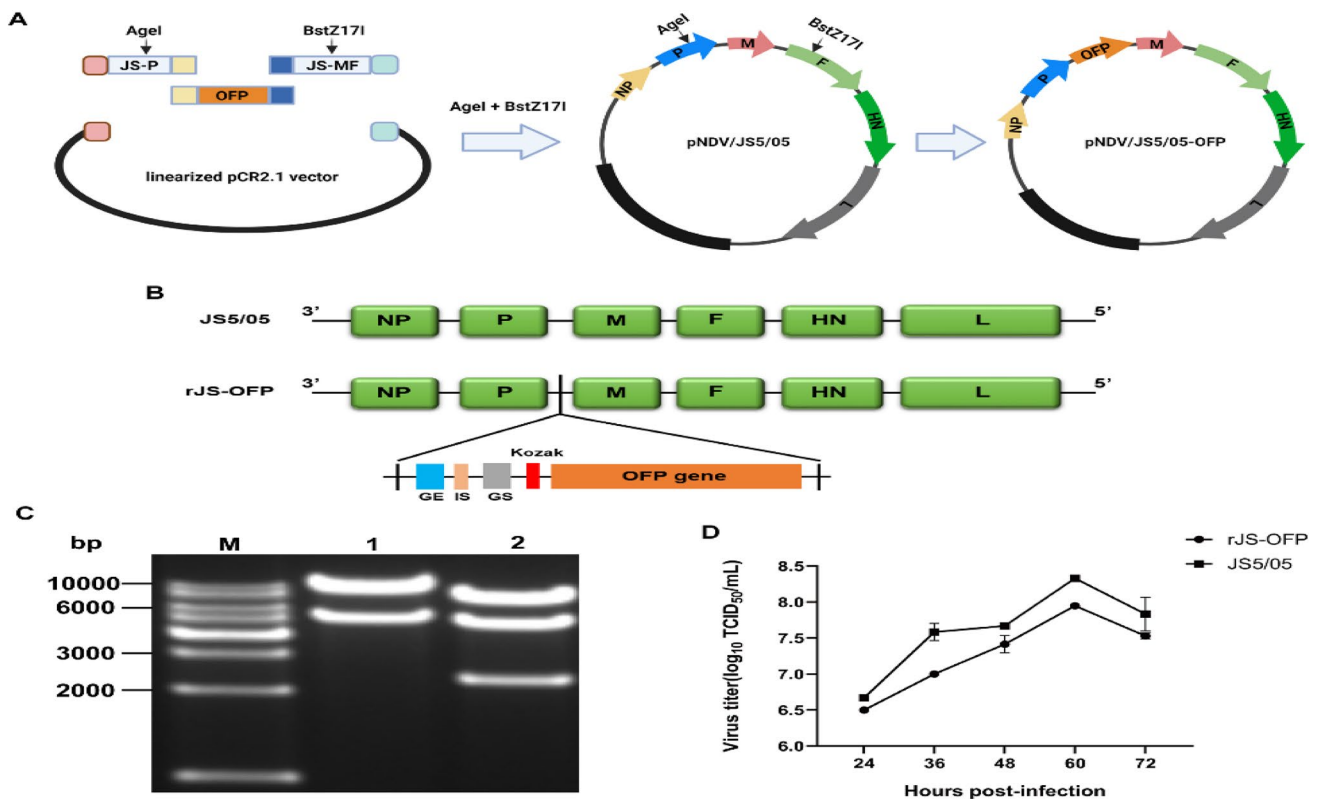
Fig.1 Generation of the recombinant NDV containing the OFP gene. **A** Illustration of the strategy for construction of the recombinant full-length cDNA containing the OFP gene. The OFP gene, two fragments from JS5/05, and the linearized pCR2.1 vector were combined using the in-fusion strategy. Then, the gene cassette was transferred to the full-length clone of JS5/05 using *Age*I and *Bst*Z17I, resulting in insertion of the OFP gene between the P and M genes. **B** Schematic illustration of the structure of the recombinant NDV. The OFP gene with the addition of the NDV transcriptional signals, including the gene end (GE), intergenic sequence (IS), and gene start (GS) as well as the Kozak motif, was expressed as an additional transcriptional unit between the P and M gene in the NDV genome. **C** Identification of the recombinant full-length plasmids by digestion with *Nde*I. M: DNA marker; lane 1: pNDV/JS5/05; lane 2: pNDV/JS5/05-OFP digested. **D** Growth kinetics of the recombinant NDV in CEF cells. The cells were inoculated with the NDVs at a MOI of 0.01, and the supernatants were collected at 24, 36, 48, 60, and 72 h post inoculation for virus titration. The experiments were performed in duplicate and the mean values ± standard error were shown

*Bst*Z17I. The resultant recombinant FL clone was designated as pNDV/JS5/05-OFP. The sequences of primers used for plasmid construction were listed in Table 1 and the detailed information of the primers was also presented.

Rescue of the recombinant NDV

The recombinant NDV was rescued using the reverse genetics as described previously [15]. Briefly, BSR-T7/5 cells were seeded into 35-mm dishes and cultured overnight, and were used for transfection when the confluency reached 70–80%. The cells were washed with PBS for three times, and modified vaccinia virus was added to the cells for adsorption for 1 h at 37 ℃. After washing with PBS, the full-length cDNA plasmid pNDV/JS5/05-OFP and three helper plasmids were co-transfected into the cells at a ratio of 2: 2: 1: 1 using the X-tremeGENE HP DNA transfection reagent (Roche, Basel, Switzerland). Three days post-transfection,

the supernatant was harvested, and 0.2 mL was inoculated into the allantoic cavities of 9-day-old ECEs. Four days post inoculation (pi), the allantoic fluids were harvested, and the presence of virus was identified by hemagglutination (HA) test. The HA-positive allantoic fluids were propagated in 9-day-old ECEs. The recovered virus was designated as rJS-OFP.

Characterization of the recombinant NDV

To determine the genetic stability of the recombinant NDV, the virus was propagated in ECEs for five passages, and the presence of the OFP gene in NDV genome was detected using RT-PCR. Virus yield was analyzed by measuring HA activity, 50% tissue culture infectious dose ($TCID_{50}$) in CEF cells, and 50% embryo infectious dose (EID_{50}). In addition, to assess the impact of OFP gene insertion on NDV replication, the growth kinetics of the recombinant virus and

Table 1 The primers used in this study

Primer	Sequence (5′to 3′)[a,b]
JS5/05-P-F	CGCCAGTGTGCTGGAATTCGGCTTTAAACTCTCACAACCGGTGC
JS5/05-P-R	GGTGGCTTCTACCCGTATTTTTTCTTAATTAATAATGCTGCTGGATTGGTTGC
OFP-F	<u>TAAGAAAAAA</u>**T***ACGGGTAGAA*GCCACCATGTCTAAGGGAGAAGAAAA
OFP-R	TTGATTCTACCCGTATTTTTTCTTATCGTTACTTGTAAAGTTCATC
JS5/05-MF-F	TAAGAAAAAATACGGGTAGAATCAAAGTGCCTCGATTGCAC
JS5/05-MF-R	ATATCTGCAGAATTCGGCTTTGAGCAGAGTAGTCAGTGTT

[a]Gene end sequence (underlined), intergenic sequence (bold), gene start sequence (italic)

[b]Homologous sequences (lower case) for in-fusion cloning

parental virus was determined in CEF cells. In brief, the cells were seeded in six-well plates and cultured in the complete medium (M199 medium containing 4% FCS) overnight. The cells were then inoculated with the viruses at a multiplicity of infection (MOI) of 0.01. After virus absorption for 1 h, the inoculum was removed, and the maintaining medium (M199 medium supplemented with 1% FCS) was added. The culture supernatants were harvested at 24, 36, 48, 60, and 72 h pi, and the virus content in the supernatants was determined. The collected supernatants were tenfold serially diluted in the maintaining medium and inoculated into CEFs. At 72 h pi, the presence of the virus in the culture supernatants was detected using HA assay and $TCID_{50}$ titers were calculated. Additionally, the virulence of the recombinant virus was evaluated by standard MDT test [16]. Briefly, the recombinant and parental virus were serially diluted and 0.1 mL of the 10^{-6} to 10^{-10} dilutions was inoculated into 9-day-old ECEs, five eggs per dilution. The eggs were monitored three times a day for 5 days. The death time of each chicken embryo was recorded and the MDT was calculated.

Expression of the OFP by the recombinant NDV

To detect the expression of the OFP protein, 1×10^5 of DF1 cells in a 12-well plate were inoculated with the recombinant virus and parental virus at a MOI of 1. At 36 h pi, the cells were fixed with 4% paraformaldehyde at room temperature for 10 min and then incubated with a monoclonal antibody against the NDV HN protein as the primary antibody at 37 ℃ for 1 h. The cells were then incubated with FITC-conjugated goat anti-mouse antibody (TransGen Biotech, Beijing, China) as the secondary antibody at 37 ℃ for 1 h. Cell nuclei were stained with DAPI (Beyotime). The cells were examined under a fluorescence microscope to monitor the expression of the OFP and NDV HN protein.

Pathogenicity of the recombinant NDV in chickens

To assess the pathogenicity of the recombinant virus in vivo, twenty-seven 5-week-old SPF white leghorn chickens were randomly divided into three groups of nine chickens per group. Chickens were inoculated with 10^5 EID_{50} of the recombinant virus and parental virus by the intranasal and intraocular routes. The animals were monitored daily and clinical signs were scored as following: 0, normal; 1, mild symptoms (mild depression); 2, moderate symptoms (diarrhea, respiratory signs); 3, severe symptoms (marked depression, open-mouth breathing, paralysis and obvious neurological signs); 4, dead. At day 1 and 3 pi, three chickens per group were euthanatized for observation of gross pathology. In addition, the spleen, lung, and intestinal tract were collected for virus titration. The remaining three chickens of each group were observed daily for 12 days for clinical signs and mortality.

Results

Generation of the recombinant NDV containing the OFP gene

To investigate the impact of foreign gene insertion on NDV pathogenicity, the OFP gene, a widely used fluorescent reporter, was selected as a model and was inserted into the genome of NDV JS5/05. The OFP gene was cloned between the P and M genes in the FL cDNA clone of JS5/05 through a combined strategy of in-fusion and ligation (Fig. 1A, B). Digestion of the parental FL cDNA plasmid pNDV/JS5/05 with the restriction enzyme *Nde*I resulted in two fragments of 6.54 and 11.72 kb, while *Nde*I digestion of the recombinant plasmid pNDV/JS5/05-OFP produced three fragments of 2.84, 6.54, and 9.63 kb (Fig. 1C), suggesting successful insertion of the OFP gene into the NDV genome. The recombinant NDV was then rescued using reverse genetics. At day 4 post transfection, red fluorescence was detected in the transfected cells but not in the non-transfected cells (data not shown). The allantoic fluids collected from the ECEs inoculated with the transfection products had positive HA activity and the HA titer reached 8 $\log_2$ after two passages in ECEs. The

results indicated that the recombinant NDV containing the OFP gene was successfully rescued.

Biological characterization of the recombinant NDV

The genetic stability of the recombinant NDV was evaluated after five consecutive passages in ECEs. The OFP gene was amplified using RT-PCR in the genome of the recombinant virus of each passage (data not shown), indicating stable presence of the OFP gene in the NDV genome. Additionally, to determine if insertion of the OFP gene into the JS5/05 genome affects the biological properties of the virus, virus titers, growth kinetics, and virulence of the recombinant virus were measured. Virus titers of the recombinant virus, expressed as HA titer, EID_{50} and $TCID_{50}$, were comparable to those of the parental strain (Table 2). However, the MDT value of the recombinant virus was 7 h longer than that of the parental virus (Table 2), suggesting a mild attenuation of the virus in eggs. Moreover, growth kinetics revealed that the virus titers of rJS-OFP in CEF cells were lower than that of JS5/05, but the difference was within the range of 1 $\log_{10}$,

indicating comparable replication capacity between these viruses (Fig. 1D). These results indicated that insertion of the OFP gene caused no significant changes of virus replication in cells and ECEs but a mild decrease in the virulence in chicken embryos.

Expression of the OFP by the recombinant NDV

To investigate the expression of the OFP by the recombinant NDV, DF1 cells were infected with rJS-OFP and JS5/05 at a MOI of 1. Co-expression of the NDV HN protein and the OFP was assessed. At 36 h pi, intense red fluorescence was observed in the cells inoculated with rJS-OFP, whereas no red fluorescence was seen in JS5/05-infected and non-infected cells (Fig. 2). Of note, co-expression of the HN protein and the OFP was seen in almost every cell, despite a few HN-positive cells with low OFP intensity. These results showed that the OFP was expressed efficiently and stably in the cells infected with the recombinant NDV.

Pathogenicity of the recombinant NDV in chickens

The MDT test indicated that the virulence of the recombinant NDV was slightly attenuated compared to the parental virus. To assess the impact of OFP insertion on virus pathogenicity in susceptible chickens, 5-week-old SPF chickens were inoculated with 10^5 EID_{50} of JS5/05 and rJS-OFP through the intranasal and intraocular routes. The parental virus caused severe clinical signs in chickens at day 3 pi, including marked depression and respiratory signs (4/6),

Table 2 Biological characteristics of the NDVs

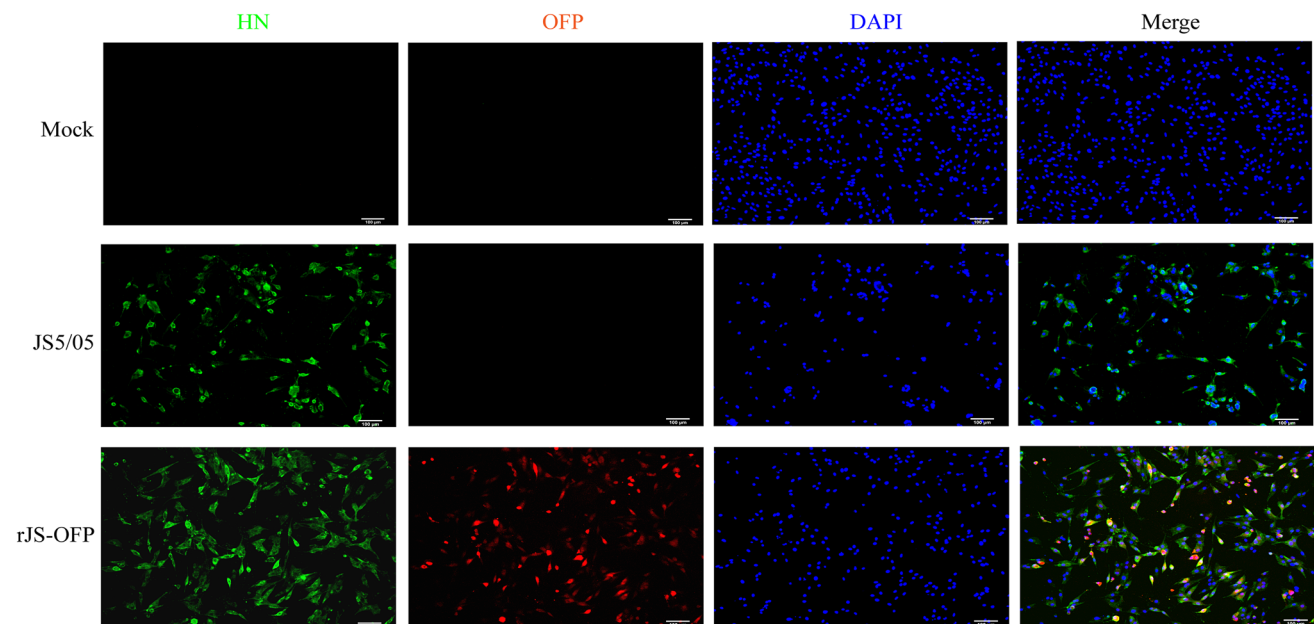| Virus	Virus titer			Virulence
	HA ($\log_2$)	$TCID_{50}/mL$ ($\log_{10}$)	EID_{50}/mL ($\log_{10}$)	MDT (h)
rJS-OFP	8	9.33	9.5	55
JS5/05	8	9.33	9.83	48

Fig. 2 Expression of the OFP by the recombinant NDV. DF1 cells were inoculated with rJS-OFP and JS5/05 at a MOI of 1. At 36 h post inoculation, the expression of the OFP and NDV HN protein was examined. Scale bar: 100 μm

Table 3 Summary of clinical signs and lesions in chickens infected with NDVs

| Group	Clinical signs	Lesions
JS5/05	Day 1–2 pi: no obvious clinical signs Day 3 pi: severe depression and respiratory signs (4/6); paralysis (2/6); marked neurological signs (2/6) Day 5 pi: all chickens died	Day 1 pi: no pathological lesions Day 3 pi: enlarged spleen (3/3); necrosis in the spleen (3/3); petechial hemorrhages in the thymus (3/3); ulceration in the intestine (3/3)
rJS-OFP	Day 1–6 pi: no obvious clinical signs Day 7 pi: mild depression (1/3) Day 11 pi: severe depression and paralysis (1/3), which was euthanized Day 12 pi: No spontaneous deaths at the end of experiment	Day 1 pi: no pathological lesions Day 3 pi: mildly enlarged spleen (1/3); mild hemorrhages in the intestine (1/3)
Mock	No clinical sings observed	No lesions observed
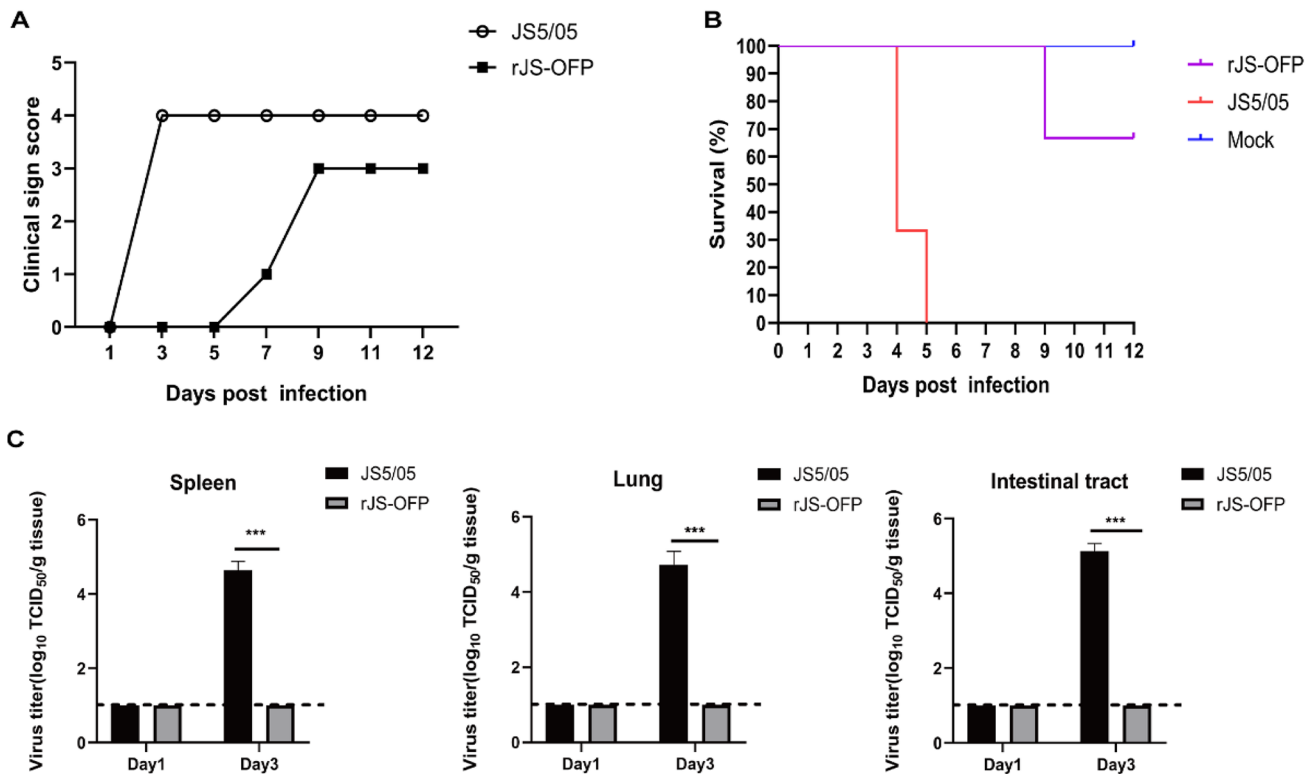
Fig. 3 Pathogenicity of the recombinant NDV in chickens. **A** Scoring of the clinical signs of the chickens after virus infection. The chickens were inoculated with the recombinant NDV and parental virus at 10^5 EID_{50} via intranasal and intraocular routes. The animals were examined daily, and the clinical signs were scored as described in the materials and method section. Mean scores of each group during a 12-day observation period were calculated and shown. **B** Survival of the chickens after virus infection. The chickens in each group were monitored daily for 12 days and the mortality was recorded. **C** Viral load in the tissues of the NDV-infected chickens. The spleen, lung, and intestinal tract of the chickens in each group were collected at day 1 and 3 post infection. Virus content was determined by inoculating the samples into CEF cells. Mean titers ± standard error of three chickens were shown. Asterisks (***) indicate significant differences ($p < 0.001$). The dotted line stands for the limit of detection of the titration assay (1.48 $\log_{10}$ $TCID_{50}/g$)

paralysis (2/6), and obvious neurological signs (2/6). At day 5 pi, all chickens succumbed to virus infection (Table 3 and Fig. 3A). In terms of pathological changes, no obvious lesions were observed in JS5/05-infected chickens at day 1 pi, and remarkable lesions were seen at day 3 pi, such as enlargement and marked necrosis in the spleen (3/3),

hemorrhages in the thymus (3/3), and necrotic ulceration in the intestine (3/3) (Table 3). In contrast, the onset of clinical signs in chickens infected with rJS-OFP was delayed, starting at day 7 pi. In addition, the severity of clinical signs was lower than that caused by the parental virus (Fig. 3A). Only mild depression was seen in one chicken from day

7 to 10 pi. At day 11 pi, severe depression and paralysis were seen in this chicken, which was humanely euthanized. Until the end of the observation period, no spontaneous death was observed (Table 3). In addition, at day 3 pi, rJS-OFP only induced mild enlargement of the spleen (1/3), petechial hemorrhages in thymus and duodenal loop (1/3) (Table 3). The parental NDV JS5/05 strain caused 100% mortality in chickens, whereas JS5/05-OFP only caused 33% mortality (Fig. 3B). Virus load measurement revealed that JS5/05 caused a systematic infection in chickens, with high virus titers recovered from the spleen, lung, and intestine (Fig. 3C). Unexpectedly, virus load of rJS-OFP in these tissues was all below the detection limit of the titration assay (1.48 $\log_{10}$ TCID$_{50}$/g) (Fig. 3C), suggesting no or very low virus replicating in chickens. These results indicated that insertion of the OFP gene in JS5/05 significantly attenuated the pathogenicity and virus replication in susceptible chickens.

Discussion

NDV is used as a vector to express genes of interest but the impact of foreign gene expression on the pathogenicity of NDV is not clear. To investigate the impact of foreign gene insertion on NDV pathogenicity in susceptible chickens, a recombinant virus expressing the OFP gene based on a virulent strain was generated using reverse genetics. Insertion of the OFP gene between the P and M gene in the NDV genome caused no significant changes in virus replication but a mild attenuation of the virulence in chicken embryos. Notably, the pathogenicity and replication of the recombinant NDV were significantly decreased in susceptible chickens compared to the parental virus. Our results suggested that in vitro characteristics associated with foreign gene insertion in NDV do not correlate to the changes of virus pathogenicity in vivo.

Most studies on the NDV vector were performed in lentogenic or modified attenuated virus backbones, and the recombinant viruses were mainly characterized by measuring in vitro replication and the virulence. The MDT test in chicken embryos and ICPI in day-old chickens were commonly used to evaluate the virulence. Independent studies reported that insertion of different foreign genes, such as the G or F gene of Nipah virus, gB or gD gene of infectious laryngotracheitis virus, between the P and M gene in La Sota backbone resulted in slight alteration in virus replication, MDT and ICPI values [17, 18]. Similar findings were also obtained in our study using P-M gene junction as the insertion site for the OFP gene. Another concern is that whether insertion of foreign genes in other sites, instead of the P-M junction, in the NDV genome, would exert distinct effects on the virus. An early study showed that insertion of the CAT gene immediately before the open reading frame of

the 3′d-proximal NP gene of La Sota strain resulted in marginal changes in the pathogenicity as determined by ICPI and virus replication in DF1 cells [8]. Zhao et al. found that GFP insertion in different genomic sites of the NDV VG/GA strain caused little effect on virus titer and the pathogenicity (MDT and ICPI), but P-M gene junction resulted in the highest fluorescence intensity [19]. Although ICPI is the golden standard to determine the virulence of NDV strains; however, ICPI values of lentogenic NDVs are low (close to 0), and thus, it is difficult to reflect the impact of foreign gene insertion on the pathogenicity based on ICPI. In addition, ICPI is measured by intracerebrally inoculating NDV in day-old chickens, which may not represent the real changes of pathogenicity in chickens under natural infection routes.

Nevertheless, studies elucidating the effect of foreign gene insertion on the pathogenicity of mesogenic or velogenic NDV strains are very limited. The first study on expression of the foreign gene in NDV vector showed that CAT gene insertion between the HN and L genes in mesogenic BC strain induced obvious attenuation in virus replication and the pathogenicity [11]. However, chicken infection experiment of the recombinant NDV was not conducted. Additionally, two teams measured the pathogenicity of a recombinant NDV expressing the GFP gene based on a velogenic ZJ1 strain (rZJ1-GFP) in chickens, and found that rZJ1-GFP is highly pathogenic (100% mortality at day 5 pi) in chickens as its parental virus [12, 13]. In contrast, Marcano et al. observed apparent attenuation of the rZJ1-GFP strain in 4-week-old chickens, with only 20% mortality at day 5 pi and survival of two chickens at day 10 pi [6]. In this study, we also showed that insertion of the OFP gene in velogenic JS5/05 strain significantly impaired the pathogenicity and virus replication in 5-week-old chickens, which correlated to the findings from Marcano's work. The reason for these conflicting results is not clear yet, which deserves further studies.

Moreover, different cytokine genes were expressed in velogenic NDV backbones to investigate the role of cytokines in regulating NDV pathogenicity. Expression of IFN-γ in ZJ1 caused significant decrease in the pathogenicity in chickens, and insertion of IL-2 and IL-4 in the same strain resulted in reduction in virus load and alteration in pathological manifestation, respectively [6, 7, 13]. It is noted that the observed changes in virus characteristics in these studies may be mainly associated with the immunoregulatory function of the cytokines. The OFP gene, without any immune regulatory activities, was inserted in the NDV genome, and thus, our results may be more related to the effect of foreign genes on the pathogenicity.

In summary, a recombinant NDV expressing the OFP gene was generated and evaluated in this study. OFP gene insertion in a velogenic NDV caused no substantial changes in virus replication and only a mild attenuation of virulence

in chicken embryos. Chicken infection studies revealed that insertion of the OFP gene resulted in significant decrease in the pathogenicity and replication in susceptible chickens. Therefore, in vitro assessment of the recombinant NDV expressing the foreign genes did not correlate to the real features of the virus in vivo. Our findings provided further understanding of evaluation of the pathogenicity of NDV.

Author contributions ZH and XL conceptualized the project. JN, QC and JD developed the methodology and conducted the experiments. TL and YC analyzed the experimental data. JH and SH revised and edited the manuscript.

Declarations

Competing interest The authors declare no competing interests.

Conflict of interest The authors declare no conflicts of interest.

Ethical approval All animal experiments were approved by the Jiangsu Administrative Committee for Laboratory Animals (Permission number: SYXK-SU-2007–0005), and complied with the guidelines of Jiangsu laboratory animal welfare and ethics of Jiangsu Administrative Committee of Laboratory Animals. Experiments involving virulent NDVs were performed in animal biosafety level-3 facilities.

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