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ORIGINAL PAPER

# Establishment of reverse genetics for genotype VII Newcastle disease virus and altering the cell tropism by inserting TMPRSS2 into the viral genome

**Jing Wu[1] · Rongguang Lu[2] · Jigui Wang[1] · Jun Su[1] · Chenchen Gu[1] · Qianqian Xie[1] · Hui Zhu[1] · Jun Xiao[3] · Weiquan Liu[1]**

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## Abstract

Newcastle disease (ND) is the most important infectious disease in poultry, which is caused by avian orthoavulavirus type 1 (AOAV-1), previously known as Newcastle disease virus (NDV). In this study, an NDV strain SD19 (GenBank accession number OP797800) was isolated, and phylogenetic analysis suggested the virus belongs to the class II genotype VII. After generating wild-type rescued SD19 (rSD19), the attenuating strain (raSD19) was generated by mutating the F protein cleavage site. To explore the potential role of the transmembrane protease, serine S1 member 2 (TMPRSS2), the TMPRSS2 gene was inserted into the region between the P and M genes of raSD19 to generate raSD19-TMPRSS2. Besides, the coding sequence of the enhanced green fluorescent protein (EGFP) gene was inserted in the same region as a control (rSD19-EGFP and raSD19-EGFP). The Western blot, indirect immunofluorescence assay (IFA), and real-time quantitative PCR were employed to determine the replication activity of these constructs. The results reveal that all the rescued viruses can replicate in chicken embryo fibroblast (DF-1) cells; however, the proliferation of raSD19 and raSD19-EGFP needs additional trypsin. We next evaluated the virulence of these constructs, and our results reveal that the SD19, rSD19, and rSD19-EGFP are velogenic; the raSD19 and raSD19-EGFP are lentogenic; and the raSD19-TMPRSS2 are mesogenic. Moreover, due to the enzymatic hydrolysis of serine protease, the raSD19-TMPRSS2 can support itself to proliferate in the DF-1 cells without adding exogenous trypsin. These results may provide a new method for the NDV cell culture and contribute to ND's vaccine development.

**Keywords** NDV · Reverse genetics · TMPRSS2 · Cell culture

## Introduction

Newcastle disease (ND) is a globally spread disease for domestic or wild birds, which was named for the first isolation in Newcastle, England, in 1927 [1]. This highly epidemic disease is caused by avian orthoavulavirus 1 (AOAV-1), commonly known as Newcastle disease virus (NDV), and belongs to the genus *Orthoavulavirus* in the family *Paramyxoviridae* [2]. NDV has a negative-sense, single-stranded RNA genome of approximately 15.2 kb, including six open reading frames (ORFs) [3, 4], sequentially coding for six structural proteins: nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (L), together with two non-structural proteins V and W by the mechanism of RNA editing [5].

Based on the full F gene sequence, NDV can be divided into 2 classes, with class I containing 3 genotypes and class

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✉ Jun Xiao
xxxjjj6288@sina.com

✉ Weiquan Liu
weiquan8@cau.edu.cn

[1] State Key Laboratory of Agrobiotechnology, Department of Biochemistry and Molecular Biology, College of Biological Sciences, China Agricultural University, No. 2 Yuanmingyuan West Road, Beijing 100193, China

[2] School of Medicine, The Chinese University of Hong Kong, Shenzhen (CUHK-Shenzhen), Shenzhen 518172, Guangdong, People's Republic of China

[3] Department of Geriatrics, The Eight Medical Centre, Chinese PLA General Hospital, Beijing, China

II containing 21 genotypes (I–XXI) [6]. NDV can also be divided into three pathotypes of increasing virulence: lentogenic, mesogenic, and velogenic [7]. The assessment of virulence includes the mean death time (MDT), intracerebral pathogenicity index (ICPI), and intravenous pathogenicity index (IVPI) [8]. The molecular basis for virulence lies in the F protein, the formation of which requires cleavage of precursor $F_0$ protein into $F_1$ and $F_2$ between the amino acid sites 116 and 117 [9]. The F protein cleavage sites of mesogenic and velogenic strains are $^{112}$R/G/K-R-Q/K/R-R/K-K/R-F$^{117}$, whereas that of lentogenic strains are $^{112}$G-K/R-Q-G-R-L$^{117}$ [10]. The former is multibasic and can be cleaved by intracellular furin-like proteases, whereas the latter is monobasic and requires cleavage by trypsin-like proteases extracellularly [9]. Because of the ubiquitous nature of furin-like proteases, infection with velogenic strains is generally fatal [11].

The transmembrane protease, serine S1 member 2 (TMPRSS2), is a type II transmembrane serine protease that shows high expression in epithelial cells of the respiratory and digestive tracts [12, 13]. TMPRSS2 is considered a novel host-directed drug target against SARS-CoV-2 via promoting both ACE2 acceptor and spike glycoprotein cleavage [14, 15]. Moreover, it has been shown that overexpression of TMPRSS2 by DF-1 (UMNSAH/chicken fibroblast) cells or MDCK (Madin–Darby canine kidney) cell line enhances influenza viral titer by cleaving $HA_0$ protein in the absence of exogenous trypsin [16, 17].

In this study, a new velogenic NDV strain (SD19) was isolated, and the topology of phylogeny suggested the strain SD19 belongs to the class II genotype VII. Subsequently, the reverse genetics system of SD19 was constructed (rSD19). After building the attenuated recombinant strain raSD19, we investigated whether inserting chicken TMPRSS2 into raSD19 can permit viral growth in cell culture without additional trypsin, which can alter the cell tropism and develop a new method for virus proliferation.

## Materials and methods

### Sample collection, isolation, and sequencing

Specific tissues of symptomatic chickens were obtained from a poultry farm with an ND outbreak in Shandong province in 2019; after grinding and centrifuging, the supernatant was taken and injected into 9-day-old specific pathogen-free (SPF) embryonated chicken eggs to isolate the virus and incubation at 37 °C with the embryo's death time concentrated between 24 and 48 h. The plaque cloning was performed three times to obtain purified viruses using CEF (chicken embryo fibroblasts) cells and 1% low-melting point agar. The viruses were incubated for 1.5 h before the addition of 1% low-melting point agar with MEM containing 2% FBS. The SPF embryos were used for viral proliferation. After that, total RNA was extracted from hemagglutination (HA)-positive allantoic fluid using an RNA extraction kit (Tiangen) and reverse transcribed employing FastKing gDNA Dispelling RT SuperMix (Tiangen). Then, ten cDNA fragments with overlap sequence ends underwent PCR amplification (the length of each one is shown in Fig. 3) and were cloned into pMD-19 T vectors to acquire the sequences determined by Sanger sequencing (Sangon). The PCR was performed using PrimeSTAR Max Premix (Takara) as the following procedure: 98 °C for 5 min, followed by 30 cycles of 98 °C for 10 s, 55 °C for 10 s, 72 °C for the 20 s, and final elongation for 5 min and the primers used are listed in Table S1. The DNASTAR Lasergene software was used to assemble the complete genome.

### Phylogeny

Eighty NDV F gene complete nucleotide sequences (1662 nucleotides) accessed from the GenBank (Table S2) were involved in the phylogenetic analysis. The analyses were conducted in MEGA11 and performed by the maximum likelihood method based on the general time reversible model with 500 bootstrap replications [18]. The codon positions included were 1st, 2nd, 3rd, and noncoding, with elimination positions less than 95% site coverage. The gaps and missing data were partial deletions. Figtree software (http://tree.bio.ed.ac.uk/software/figtree) was employed to annotate the evolutionary tree.

### Plasmids construction

The plasmid pBluescript II SK (+) (Stratagene, a generous gift from Dr. Agata Fazzio) was used as the backbone of the full-length antigenomic isolated NDV cDNA clone pB-rSD19. The ten fragments were ligated by overlap PCR to form three longer ones and cloned into pMD-19 T. Employing seamless cloning (In-Fusion® Snap Assembly, Takara), the entire genomic RNA headed by a T7 promoter and tailed with a hepatitis delta virus ribozyme (HdvRz) sequence was inserted into the vector. Three helper plasmids expressing N, P, and L were constructed (pEMC-N, pEMC-P, pEMC-L) according to the methods of Dr. Li [19]. Plasmid pB-raSD19 containing the antigenomic of the rescued virus raSD19 was constructed using Mut Express MultiS Fast Mutagenesis Kit V2 (Vazyme). Other plasmids of the reverse genetics system pB-rSD19-EGFP, pB-raSD19-EGFP, and pB-raSD19-TMPRSS2 were constructed by seamless cloning. The cDNA of chicken TMPRSS2 (XP_046765667) was synthesized and cloned into p3xFLAG-CMV-14 by restriction enzyme digestion and ligation.

### Cell culture, virus growth, and virus rescue

DF-1/UMNSAH, chicken fibroblast cells were maintained in high-glucose DMEM (Gibco) containing 10% fetal bovine serum (VivaCell) at 37 °C in an atmosphere of 5% $CO_2$. The T7 RNA polymerase high-expressing cells, BHK-T7, derived from BHK-21 (baby hamster kidney) cells, had been generated from a previous study in this laboratory [19] and maintained under the same conditions as DF-1 cells. Viruses were passaged in SPF-embryonated chicken eggs at 9–11 days of age. As for the virus rescue, Lipofectamine 3000 (Thermo Fisher) was used to conduct the transfection (Fig. 2). After seeding BHK-T7 cells in a six-well plate to 60–70% confluence, 5-μg pB-rSD19, 1-μg pEMC-N, 0.5-μg pEMC-P, and 1-μg pEMC-L were co-transfected according to the manufacturer's instructions; 2 days later, the cells were resuspended with trypsin and transferred into a 10 $cm^2$ plate. Cytopathic effects (CPE) were observed within 2–3 days. The methods of producing other rescued viruses were the same as above. The lentogenic raSD19 and raSD19-EGFP grew in the DF-1 cells or BHK-T7 cells, with the addition of 5-μg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) trypsin (Sigma). The experiments on the NDV were conducted in the laboratory of biosafety level 3 in China Agricultural University, using the biological safety cabinet.

### Viral pathogenicity assessment

Viral $TCID_{50}$ was determined in 96-well plates with tenfold serial dilution infecting DF-1 cells containing 1% FBS. Cytopathic effects were observed for 5 days, and titers were determined by the Reed and Muench method [20]. MDT and ICPI were determined to assess viral virulence. The viruses were serially diluted tenfold in phosphate-buffered saline (PBS) and injected into groups of 5 9-day-old SPF chick embryonated eggs. Following continuous observation for 120 h, the average time of death at the highest virus dilution was taken as the MDT of the virus. For determination of the ICPI, groups of 8 1-day-old SPF chicks were inoculated intracerebrally with 50-μL tenfold serial dilutions of virus followed by 8 days of observation (death scored 2, sickness scored 1, health scored 0), with the weighted mean score calculated as the ICPI [21]. The isolation devices were used for chick experiments, and a series of bio-containment precautions were taken to ensure that no biosafety violations occurred.

### Quantitative real-time PCR

RNA was reverse transcribed by FastKing gDNA Dispelling RT SuperMix (Tiangen). The real-time PCR was performed in qTOWER-2.2 (Jena) using MagicSYBR Mixture (CWBIO) described by the manufacturers and with primers listed in Table S3. Each sample was tested in triplicate; the results were calculated by the $2^{-\Delta\Delta Ct}$ method [22]. Results were calculated as the mean ± SD of three separate experiments, and the statistical analyses were performed by student's t test using GraphPad Prism software (version 9.4.1).

### Western blot

Total proteins with cells and viruses were extracted using RIPA lysis buffer (Solarbio) containing 1-mM PMSF. After incubation on ice for 20 min and centrifugation, supernatants were subjected to SDS-PAGE and then transferred to the 0.45-μm PVDF membranes by wet transfer. Following blocking with 5% skimmed milk and incubation with different primary and secondary antibodies, specific proteins were detected by enhanced chemiluminescence (Beyotime) with a chemiluminescent imaging system (Tanon 5200). The virus serum polyclonal antibody was prepared by our laboratory.

### Indirect immunofluorescence assay (IFA)

Infected with viruses for 24 h, following fixation in 4% paraformaldehyde, cells were incubated with the viral polyclonal antibody for 1 h, followed by a FITC-conjugated chicken secondary antibody (Abcam). Fluorescence was observed using a fluorescence microscope (Olympus).

## Results

### Amplification of NDV complete genome and amino acid substitutions

Ten fragments were amplified using specific primers. After Sanger sequencing, all sequences were assembled, the genome structure was obtained, and the sequence was submitted to GenBank with accession number OP797800. For convenience, SD19 is used to denote this strain in this article. The strain's F protein cleavage site is RRQKRF, typical of velogenic strains. The F protein is the most crucial virulence determinant [9], and the HN protein plays an essential role in recognizing the viruses and host cells [23]. Accession number OP797800. For convenience, SD19 is used to denote this strain in this article. The strain's F protein cleavage site is RRQKRF, typical of velogenic strains. The F protein is the most crucial virulence determinant [9], and the HN protein plays an essential role in recognizing the viruses and host cells [23]. When comparing the consensus sequence of the F and HN proteins with the reference strains used (Tables S4 and S5), the following amino acid substitutions were found: M14T, A75T, V520A, and R552K in F and S77G, G124D, K321R, E347K, G362A, E384R, and T443M in HN. Among these substitutions, Residues 520 of F located

in the transmembrane region (TM) [24], with residue 77 of HN in the heptad repeat region A (HRA) of the stalk region of HN protein [25].

## Phylogeny

The maximum likelihood method and the general time reversible model were used to conduct the phylogenetic analysis based on the full length of the F gene of SD19 and 79 other published sequences. The NDV strain isolated in this study, SD19, showed 98.98% identity with Ch/SD/672/12 and clustered within the genotype VII, subtype VIIj (Fig. 1).

## Rescue of rSD19

Ten segments were merged into three large segments using overlap PCR. Through seamless cloning, the whole antigenome RNA headed by a T7 promoter and tailed with an HdvRz sequence was inserted into the backbone vector (Fig. 2). Figure 3A shows a genetic marker distinguishing the original and rescued viruses. Five days after the co-transfection to BHK-T7 cells, a noticeable cytopathic effect was observed, indicating viral replication (Fig. 3B), and both the BHK-T7 (left, $F_0$) and DF-1 (right) cells can produce syncytia (shown by red arrows) which is a typical identification of velogenic NDV viruses. IFA and Western blot also confirmed the expression of viral proteins (Fig. 3C, D). Then the growth curves were measured, which showed identical growth characteristics of the original and rescued viruses (Fig. 3E). The above results proved the successful rescue of rSD19.

## Genetic modification of rSD19

Next, we engineered the rescued virus. First, we replaced the F protein cleavage site of the rescued virus with the site

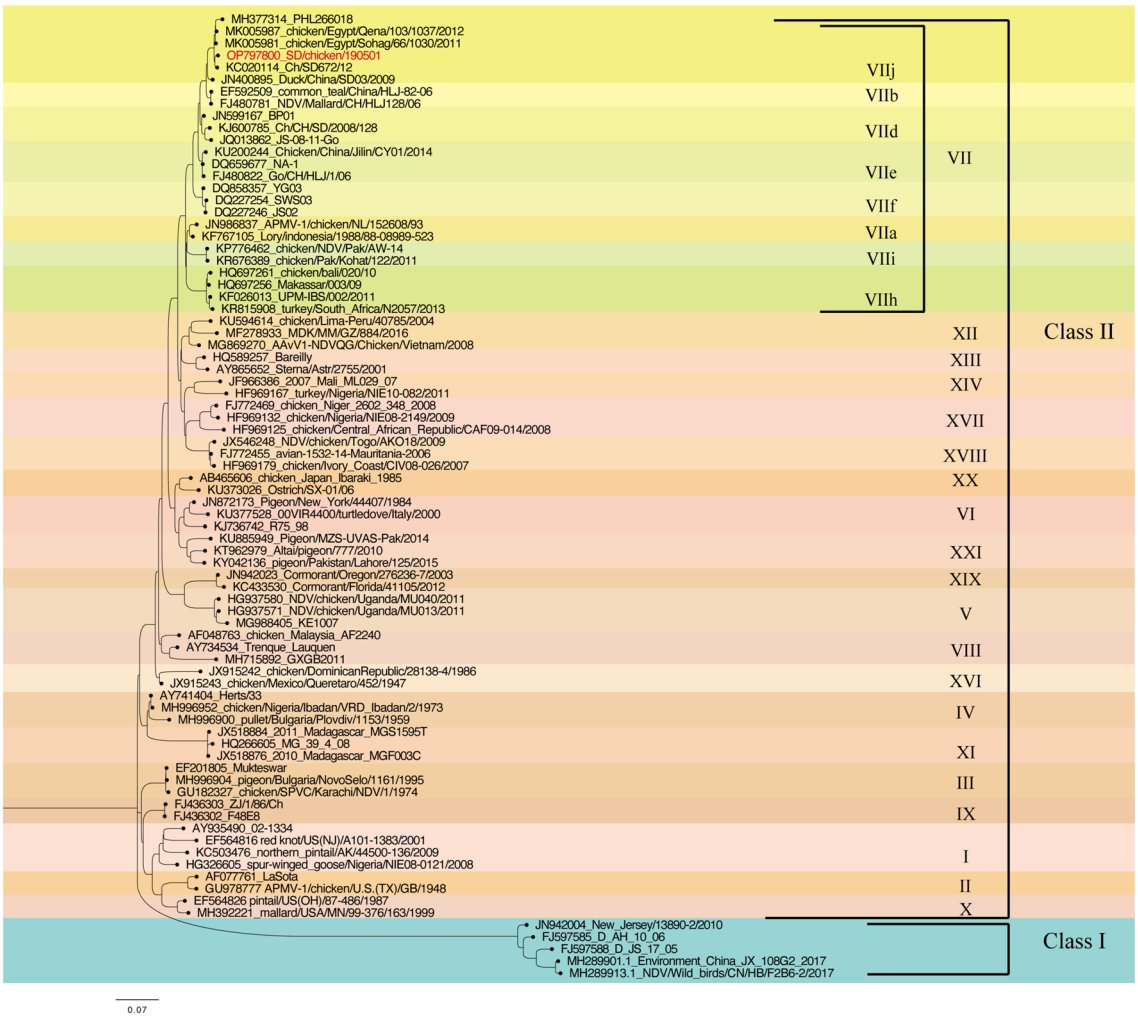


**Fig. 1** Phylogenetic analysis of the complete F gene sequences of NDV. The ML tree was constructed based on 80 sequences using MEGA 11 [18]. The sequence obtained in this study is identified in red

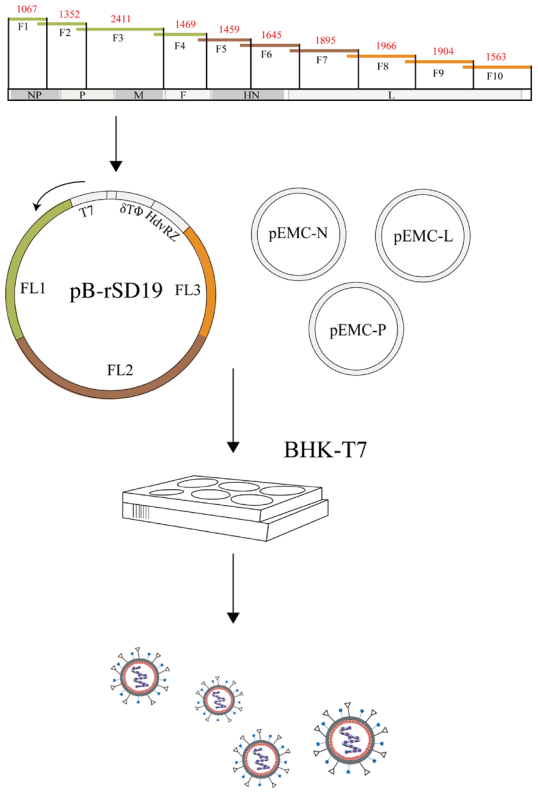


**Fig. 2** Schematic of plasmid construction and virus recovery. The ten fragments were amplified and assembled into three longer ones and different colors mean the relative location in the pB-rSD19. Then BHK-T7 cells were co-transfected with the main plasmid pB-rSD19 and three helper plasmids (pEMC-N, pEMC-P, pEMC-L) expressing N, P, and L proteins. The appearance of CPE within 5 days indicated virus generation

corresponding to the LaSota strain to change the virus virulence, thus creating the raSD19. Next, rSD19-EGFP and raSD19-EGFP were produced to verify the location of the exogenous protein. The changing and inserting sites of the rescued viruses' genome are indicated in Fig. 4C. Since only by adding exogenous trypsin can the lentogenic virus grows in DF-1 cells, we insert TMPRSS2 into the antigenomic cDNA between P and M to help the virus gain the growth ability. The chicken TMPRSS2 protein contains 486 amino acids, and AlphaFold2 [26] was used to mimic the structure of the protein (Fig. 4A); different colors represent different domains predicted. Expression of the protein in vitro showed that the size of the protein was about 54 kDa, and other splicing forms were also detected (Fig. 4B). The relative EGFP and TMPRSS2 mRNA levels of raSD19-EGFP and raSD19-TMPRSS2 are shown in Fig. 4D, indicating quite similar mRNA levels of EGFP and TMPRSS2. And the Western blot of the five rescued viruses also verified the viral protein production (Fig. 4E). Growth curves of the five rescued viruses were compared; the result is shown in Fig. 4F, from which we can observe that the EGFP insertion does not significantly impact the virus growth in DF-1 cells. Whereas, changing the F protein cleavage site makes the raSD19 and raSD19-EGFP can only grow under the exogenous trypsin-added conditions. However, the raSD19-TMPRSS2 shows the same growth characteristics in DF-1 cells with raSD19 and raSD19-EGFP, even without exogenous trypsin.

### Biological characteristics of the isolated and rescued viruses

The MDT and ICPI are usually used to measure the virulence with the criteria that for lentogenic strains, MDT > 90 h, ICPI < 0.7; the mesogenic strains, MDT, 60–90 h, ICPI, 0.7–1.5; and the velogenic strains, MDT < 60 h, ICPI > 1.5. The pathogenicity indexes in Table 1 assessed the biological characteristics of the isolated and rescued viruses. The HA titer of the SD19, rSD19, rSD19-EGFP, raSD19, raSD19-EGFP, and raSD19-TMPRSS2 were $2^8$, $2^8$, $2^8$, $2^{11}$, $2^{11}$, and $2^{10}$, respectively. The MDT values of SD19, rSD19, and rSD19-EGFP were close at 51 h, 58 h, and 56 h, and the same went for ICPI (1.86, 1.70, 1.81), which indicates the three viruses velogenic. As for raSD19 and raSD19-EGFP, the HA titer, MDT, and ICPI were quite close, indicating the two viruses lentogenic. The raSD19-TMPRSS2 shows a shorter MDT (98 h) and higher ICPI (1.39) than the two lentogenic viruses, indicating some mesogenic features of the strain.

### raSD19-TMPRSS2 supports virus growth without exogenous trypsin

As shown in Fig. 5A by the IFA results, the viral proteins of raSD19-TMPRSS2, rSD19, and raSD19 were successfully expressed. Additionally, there were obvious syncytia by raSD19-TMPRSS2 (shown in the amplified pictures), which indicates that the TMPRSS2 can significantly cleave the F protein of the viruses. The fluorescence image also confirmed the EGFP protein expression (Fig. 5B). Besides, due to the monobasic cleavage site of the attenuated viruses raSD19 and raSD19-EGFP, these two viruses can only occur cytopathic effect in the circumstances of trypsin added, and instead of syncytia, only cells casting off can be observed.

## Discussion

Since being reported in 1926, ND has been an enormous threat to poultry production worldwide. The last nine decades have witnessed four NDV outbreaks, with the most recent outbreak beginning in the 1980s and mainly caused by the viruses of the VII genotype [27–31]. Vaccination is required to prevent the devastating outbreak of ND, but the conventional vaccine from genotype II

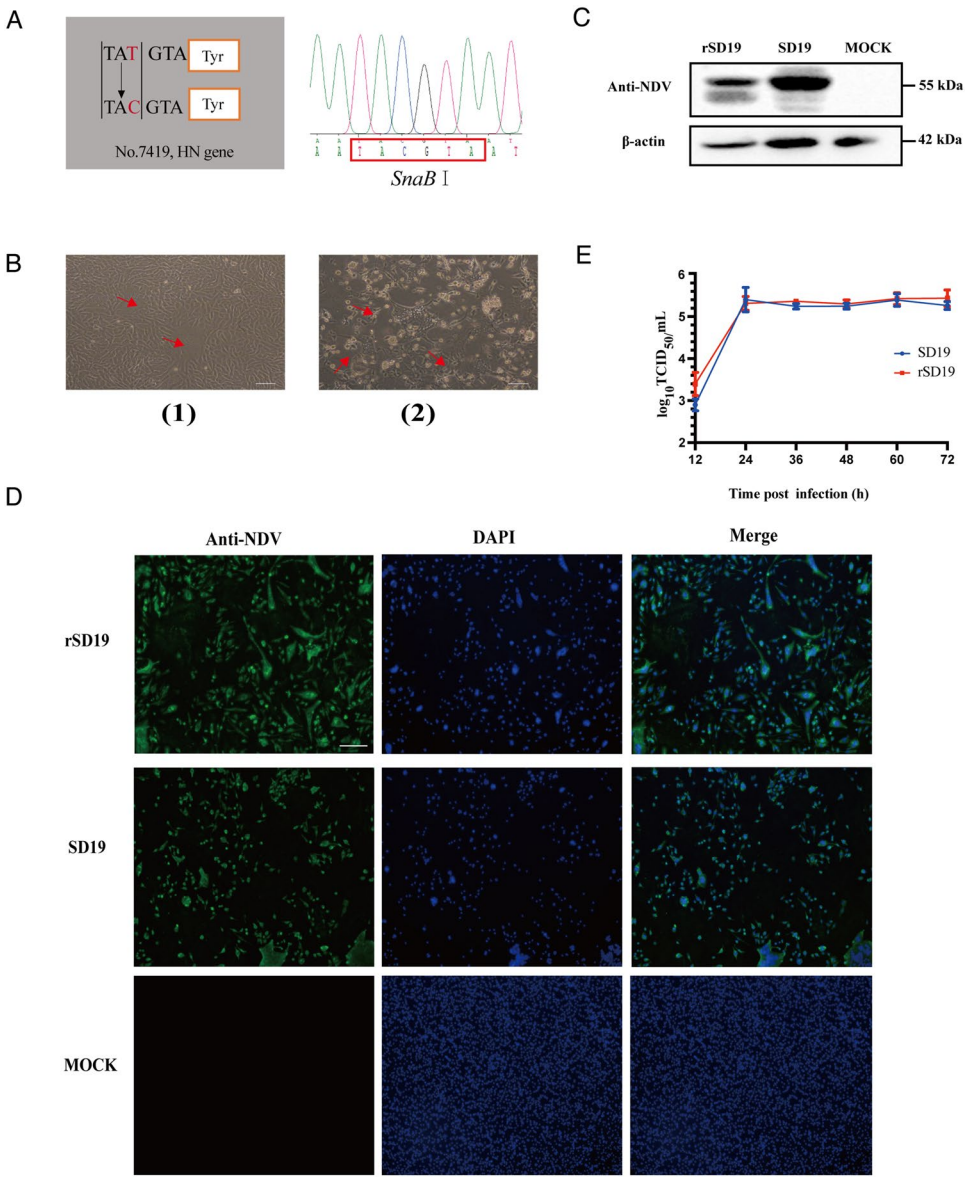


**Fig. 3** Rescue identification of rSD19. **A** The mutating position of rSD19 is located at position 7419 in the genome of the HN gene. **B** CPE of BHK-T7 (1) ($F_0$) and DF-1 (2) cells. Red arrows indicate syncytia, Bars = 100 μm. **C** Western blot showing viral protein production. **D** IFA of DF-1 cells infected with the rescued virus and originally isolated virus at an MOI of 0.1, 24 h after infection. Bars = 100 μm. **E** Growth curves of SD19 and rSD19 on DF-1 cells. The cells were infected at 0.01 MOI, and the $TCID_{50}$ of the viruses was determined after the samples were collected at 12-h intervals. Assays were performed in triplicate. Each number represents the mean of three separate experiments

cannot prevent heterologous virus shedding [32]. Here, we isolated an NDV strain SD19 and acquired the whole-genome sequence through phylogenetic tree analysis, the SD19 belongs to genotype VII, which is now widespread in Asia and Africa [33].

Reverse genetics is a powerful tool to develop the genotype-matched virus by changing the F protein cleavage site of the virulent strains from polybasic to monobasic [34–36], so after the successful rescue of rSD19, we altered the F protein cleavage site to construct the attenuated recombinant virus raSD19, and the MDT and ICPI indicated the virus lentogenic. Subsequently, the rSD19-EGFP and raSD19-EGFP were also rescued to verify the position of the inserting sites (between the P and M genes) and the potential of NDV as a vector for expressing foreign proteins as a vaccine candidate [37]. The results of biological characteristics assays showed that the insertion of EGFP does no obvious significance to the MDT and ICPI.

NDV stocks are usually propagated in SPF chicken eggs, which can get a high virus titer but have some drawbacks, such as high cost, time consumption, and labor intensity [38]. Based on the successful recovery of the rSD19, rSD19-EGFP, raSD19, and raSD19-EGFP, we wondered if directly inserting chicken TMPRSS2 complete coding sequence into the reverse genetics system of raSD19 can help the attenuated virus gain the ability of growth in DF-1 cells, and the results showed that raSD19-TMPRSS2 could grow in DF-1 cells without exogenous trypsin.

Then the biological characteristics of the total five rescued viruses were measured. The MDT and ICPI of SD19, rSD19, and rSD19-EGFP indicate that the three viruses were velogenic. The raSD19 and raSD19-EGFP show higher HA

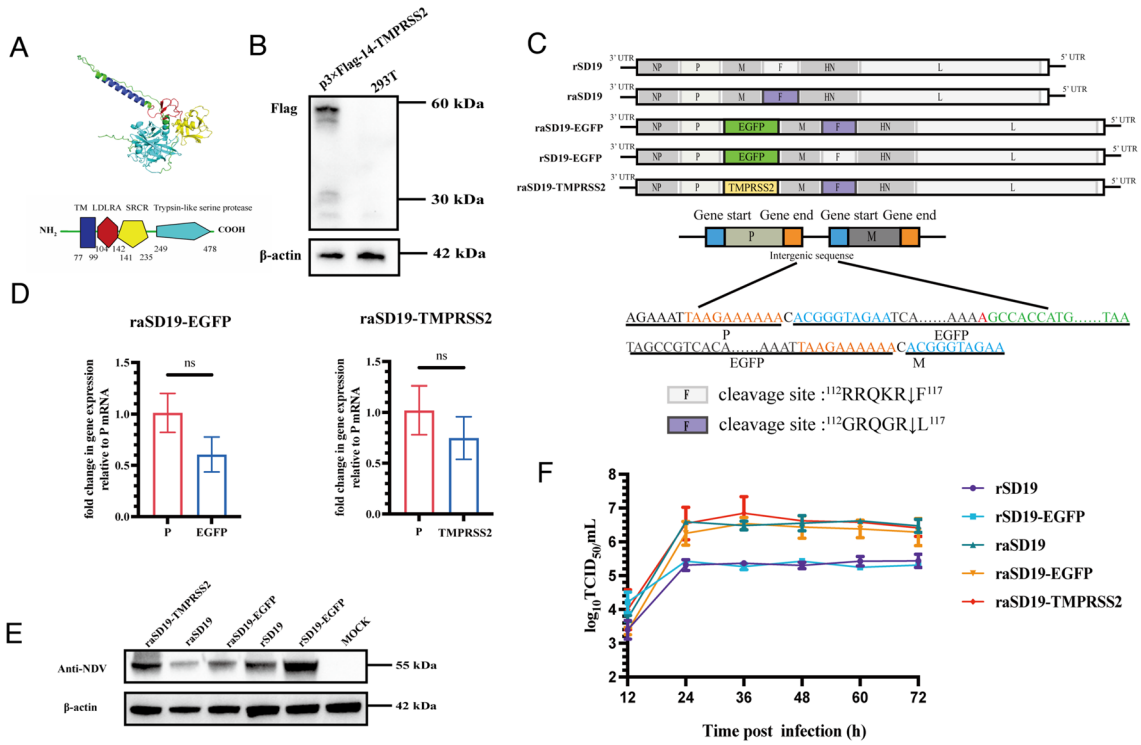


**Fig. 4** The construction of reverse genetics system of other rescued viruses. **A** Protein structure simulation of TMPRSS2 by AlphaFold2. The colors show by domains. TM transmembrane, LDLRA low-density lipoprotein receptor domain class A, SRCR scavenger receptor cys-rich. **B** In vitro expression of TMPRSS2. 293 T cells was transfected with p3 × Flag-TMPRSS2; 48 h later, protein expression was detected by Western blot. **C** Schematic of plasmid construction. The F gene marked in purple represents changing the F protein cleavage site of the SD19 into the site of the LaSota strain. The EGFP coding sequence was headed by the gene start, 5′ UTR, and Kozak sequence and tailed with 3′UTR and gene end sequence. Seamless cloning was used to conduct the insertion of the EGFP. The insertion of TMPRSS2 was performed by the same methods as raSD19-EGFP. **D** The mRNA expression level of EGFP (left) and TMPRSS2 (right) relative to P mRNA. The raSD19-EGFP or raSD19-TMPRSS2 infected DF-1 cell with 0.01 MOI; 36 h after, the RNA of infected cells was extracted, and RT-qPCR was performed to monitor the mRNA of EGFP and TMPRSS2 relative to P mRNA (Both the red and the blue columns represented the virus-infected cells, and the x-axis showed the name of the measured mRNA). Results are presented as mean ± SEM, ns, no significance. **E** Western blot of the five strains of rescued viruses, the DF-1 cells were infected by viruses at 0.01 MOI. 48-h post-infection, expression of virus protein was observed by Western blot. **F** The growth curves of rSD19, raSD19, rSD19-EGFP, raSD19-EGFP, and raSD19-TMPRSS2 on DF-1 cells. The cells were infected at 0.01 MOI, and the $TCID_{50}$ of the viruses was determined after the samples were collected at 12-h intervals. Assays were performed in triplicate. Each number represents the mean of three separate experiments

**Table 1** Pathogenicity indexes of SD19 and related rescued viruses

| Virus | HA | MDT (h) | ICPI |
|---|---|---|---|
| SD19 | $2^8$ | 51 | 1.86 |
| rSD19 | $2^8$ | 58 | 1.70 |
| rSD19-EGFP | $2^8$ | 56 | 1.81 |
| raSD19 | $2^{11}$ | > 120 | 0.16 |
| raSD19-EGFP | $2^{11}$ | > 120 | 0.14 |
| raSD19-TMPRSS2 | $2^{10}$ | 98 | 1.39 |

*HA* hemagglutination titer, *MDT* mean death time (hours) required for the minimum lethal dose (MLD) to kill all embryonated eggs for a given dilution, *ICPI* intracerebral pathogenicity index of 1-day-old chicken

titers, with the typical lentogenic indexes in MDT and ICPI. As for the raSD19-TMPRSS2, the virus shows a lentogenic characteristic in MDT, whereas shows a mesogenic feature in ICPI [39], which we speculated the expression of TMPRSS2 may promote the F protein cleavage thus producing more virulence to the 1-day-old chicken. Besides, the assessment method of ICPI is intracerebral inoculation, which is not a natural infection way of NDV, and thus the ICPI maybe not all inclusive about the virulence. Ni et al. inserted an orange fluorescent protein (OFP) into virulent NDV JS5/05 to form rJS-OFP and found a very slight increase in MDT, but when injected into 5-week-old chicken, the parental virus showed significantly more clinical signs, higher death rates, and higher virus titer in tissues [40]. So maybe the virulence of NDV is measured in many other ways and further assessments may need to operate. Moreover, the influence factors

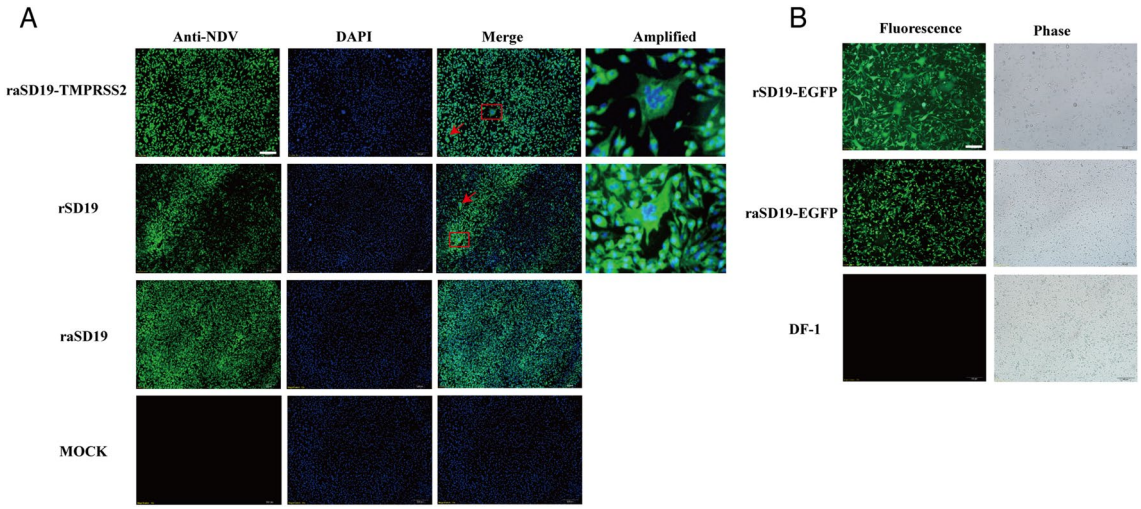


**Fig. 5** **A** IFA of raSD19-TMPRSS2, rSD19, and raSD19. The DF-1 cells were infected by viruses at 0.1 MOI, and the IFA shows the production of the viruses. Syncytia were shown in the amplified picture. The red arrows indicate the syncytia, with the boxes meaning the amplified areas. **B** The fluorescence image of the two EGFP expression rescued viruses, rSD19 and rSD19-EGFP. Bars = 100 μm

of NDV virulence are multitudinous, all six structural proteins and two non-structural proteins can influence NDV virulence, and among those, the F protein makes the most contributions to the NDV virulence according to the summary of Dortmans et al. [9]. But they discovered that the F protein of pigeon paramyxovirus type 1 (PPMV) AV324 does not determine the virulence after they changed the F gene of AV324 into the virulent Herts 33 and made no significant influence on the MDT and ICPI [41].

Studies have discovered that overexpression of TMPRSS2 in Vero cells can propagate the PEDV in the absence of exogenous trypsin [42] and influenza A viruses can be activated by TMPRSS2 [43, 44], here we constructed the raSD19-TMPRSS2 to verify the function of the protein toward NDV directly, and the rescued virus showed a higher virus titer than rSD19 and rSD19-EGFP and lower than raSD19 and raSD19-EGFP; interestingly, the ICPI of raSD19-TMPRSS2 was between the velogenic and lentogenic, which may due to the protease function of TMPRSS2 protein that particularly regains the ability of F protein cleavage.

In summary, we isolated and classified a predominant genotype VII NDV; while building the reverse genetics system of the originally isolated virus, four genetically modified rescued viruses were also built, and the rescued raSD19-TMPRSS2 obtained the ability to survive in DF-1 cells without exogenous trypsin. Although there are essential discoveries revealed by these studies, they are also limitations, the raSD19-TMPRSS2 still has higher virulence than raSD19 and how TMPRSS2 works needs to be discovered.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11262-023-01999-9.

**Author contributions** JW and WL conceived the study. JW conducted the experiments, analyzed the data, and wrote the original draft. WL and RL modified the draft. JW, JX, and JS revised the draft. CG, QX, and HZ analyzed the data and revised the draft.

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**Data availability** The authors declare that all the data are available in the article and supplementary materials.

## Declarations

**Competing interests** The authors declare no competing interests.

**Ethical approval** The experiments about chicken embryos and chickens were operated in accordance with the guidelines highlighted by the Ethics Committee for Laboratory Animal Welfare and Animal Experiments of China Agricultural University.

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