

Modification of the full-length cDNA clone of Newcastle disease virus isolated from an outbreak in the goose

LIU Yuliang¹, HU Shunli¹, ZHANG Yanmei¹, WU Yantao¹, LIU Xiufan (✉)¹,
Röemer-Oberdoerfer Angela², Veits Jutta², Lange Martina²

1. Key Laboratory of Animal Infectious Diseases, Ministry of Agriculture, Yangzhou University, Yangzhou 225009, China

2. Institute of Molecular Biology, Friedrich-Loeffler-Institutes, Federal Research Centre for Virus Diseases of Animals, D-17498 Insel Riems, Germany

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Abstract A 6.5-kb specific fragment containing the T7 promoter and the transcription vector was excised from the full-length cDNA clone of the Newcastle disease virus (NDV) strain ZJI of goose origin, and thereafter it was self-ligated to form a high quality plasmid for mutagenesis. Site-directed mutagenesis was used for inserting three additional G nucleotides (nts) into the region between the T7 promoter and the leader sequence of the NDV genome. RT-PCR was employed to amplify the *F/HN* gene fragments, and then they were ligated by the shared restriction enzyme *BsmBI*. Finally, the corresponding fragment in the mutant full-length cDNA was substituted with the new one. The sequencing results showed that the three additional G nts were successfully inserted and the mutant nts in the full-length cDNA were corrected. This study lays a good foundation for research on the reverse genetics of NDV strain ZJI.

Keywords Newcastle disease virus, goose, site-directed mutagenesis, genomic cDNA clone, modification

1 Introduction

Newcastle disease virus (NDV) is the causative agent of Newcastle disease (ND), which is widely distributed and has inflicted substantial costs to the poultry industry in China and other countries worldwide. NDV is classified as a member of the newly defined genus *Avulavirus* in the *Paramyxoviridae* family (Mayo, 2002). NDV has a nonseg-

mented, single-stranded, negative-sense RNA genome. Usually, the genomic length is 15,186 nucleotides (nts) (Krishnamurthy et al., 1998; Roemer-Oberdoerfer et al., 1999). However, we have completely sequenced the genome of ZJI, an isolate of NDV from an outbreak in geese in China, and we found that its genomic sequence consisted of 15,192 nts, which is six nts longer than the published full-length genome of NDV strains (Huang et al., 2004). It remains unclear what roles the six nts motif plays. NDV encodes six structural proteins (nucleoprotein, HN; phosphoprotein, P; matrix protein, M; fusion protein, F; hemagglutinin-neuraminidase, HN; large protein, L). Additionally, proteins (designated as V and W proteins) may also be produced by an RNA-editing event that occurs during the transcription of the *P* gene (Liu et al., 2004).

During recent years, the infection of NDV in waterfowl flocks has been reported frequently in some regions of China, which posed a great threat to goose production and other poultry industries (Xin et al., 1997; Liu et al., 2003). Several virulent NDV strains were isolated from outbreaks in geese and have been characterized pathotypically and genotypically in our laboratory in recent years (Liu et al., 2003). In the meantime, the full-length cDNA clone and three helper plasmids NP, P and L of NDV strain ZJI were also constructed (Liu et al., 2004). Thereafter, an attempt to rescue the NDV strain ZJI using the constructed plasmids was undertaken but resulted in failure. Therefore, to increase the efficiency of transcription in this study, three additional G nts were successfully inserted in the position between the T7 promoter and the leader sequence of the genomic cDNA clone using mutagenesis. In addition, the *F/HN* gene region in the full-length cDNA clone was substituted. The sequencing results indicated that the mutant nts were modified and the three G nts were inserted as expected. This study has laid a foundation for the rescue of infectious NDV.

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E-mail: xfliu@mail.yzu.edu.cn

2 Materials and methods

2.1 Virus and chicken embryo

NDV strain ZJI was isolated from an outbreak in a goose flock in Zhejiang Province, China in the laboratory and identified as the velogenic strain (ICPI: 1.89, MDT: 51.6h) (Liu et al., 2003). Nine to 11-day-old specific-pathogen-free (SPF) chicken embryos were bought from the Shandong Chicken Farm.

2.2 Plasmid and reagents

A transcription recombinant, TNDV4, containing the full-length cDNA clone of NDV ZJI was constructed by Huang Yong and colleagues; AMV, RNasin, T4 DNA ligase and CIAP were obtained from Promega; Agarose Gel DNA Extraction Kit and Expand High Fidelity PCR System were obtained from Roche; T vector kit (pCR2.1) from Invitrogen; restriction enzyme *BsmBI* was bought from Jingmei BioTec. Co. Ltd., and *NdeI*, *PsiI* and *FspAI* were obtained from BioLabs. QuickChange II XL Site-Directed Mutagenesis Kit was obtained from Stratagene.

2.3 Primer design and synthesis

Primers MPT7YF and MPT7YR were synthesized based on the sequences of the full-length cDNA clone of NDV ZJI and the transcription vector TVT7R (0,0). The primers were used in the mutagenesis of TNDV4 to insert three G nts in the position between the T7 promoter and the 5' terminal of *NP* gene. The G nts are marked bold.

MPT7YF: 5'-CGAAATTAATACGACTCACTATA**GGGA**-CCAAACAGAGAATCTG-3'

MPT7YR: 5'-CAGATTCTCTGTTTGGT**CCCT**TATAGTG-AGTCGTATTAATTTTCG-3'

The primers were synthesized by Huettner AG, Germany (related work was done in the Friedrich-Loeffler-Institute, Germany).

Two pairs of primers were designed and synthesized to amplify the entire *HN* gene and partially the *F* gene in the full-length cDNA clone for substitution in DNA samples obtained from the allantoic fluid.

HNMF1: 5'-GCCCC**TTATA**ATTTAGCTGGTGGCAATA-TGGAT-3'

HNMR1: 5'-TT**CGTCT**CGCAGTGTCAGTGGGTGAATT-GGGTT-3'

HNMF2: 5'-GG**CGTCT**CCACTGCACAAGAAGGGAAAT-ATGTAA-3'

HNMR2: 5'-TAT**TGCGC**ACATCTGGCTCCTGACTGCT-3'

The restriction site of *BsmBI* is marked bold italic, and additional nts were introduced in the 5' terminals of primers HNMR1 and HNMF2. *PsiI* and *FspAI* sites present in the original genomic sequence and marked italic were introduced

in the 5' terminals of primers HNMF1 and HNMR2. These primers were prepared by Shanghai BioTec. Co. Ltd.

2.4 Preparation of template for mutagenesis

The full-length cDNA clone TNDV4 was digested with *NdeI* and analyzed by gel electrophoresis. The digested fragments, 6.5 kb and 11.9 kb, were recovered and named TVTNDV6.5 and NDV11. The fragment TVTNDV6.5, containing the TVT7R (0,0) vector, was self-ligated in vitro. The ligated fragment was transformed into competent *E. coli* cells and the extracted plasmid DNA was identified and named TVTNDV6.5P, which was used as the template for mutagenesis.

2.5 Mutant strand synthesis reaction

The following comprised the PCR reaction system: super water (SW) 26 μ L, 10 \times reaction buffer 5 μ L, TVTNDV6.5P (10 ng / μ L) 10 μ L, MPT7YF (125 ng / μ L) 1 μ L, MPT7YR (125 ng / μ L) 1 μ L, dNTPs (10 mmol / L) 1 μ L, QuickSolution 1 μ L, and PfuUltra HF DNA polymerase (2.5 U / μ L). PCR cycles were set as follows: 95°C 1 min; 95°C 50 sec, 60°C 50 sec, 68°C 6 min, 18 cycles; 68°C extension for 7 min. The reaction tubes were placed on ice for 2 min to cool the reactions to \leq 37°C. Add 1 μ L of the *Dpn I* restriction enzyme (10 U/ μ L) directly to each amplification reaction below the mineral oil overlay using a small, pointed pipet tip. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin the reaction mixtures in a microcentrifuge for 1 min, then immediately incubate the reactions at 37°C for 1 hour to digest the parental (i.e., the nonmutated) supercoiled dsDNA. After transforming XL10-Gold Ultracompetent cells, the plasmid was extracted. The positive recombinant plasmid was named TVTNDV6.5PM and identified by sequencing.

2.6 Ligation of the full-length cDNA

Plasmid TVTNDV6.5PM was digested with *NdeI* and dephosphorylated. The purified plasmid NDV11 mentioned above was ligated with the dephosphorylated and purified plasmid TVTNDV6.5PM. After transformation, extraction and identification, we obtained the positive recombinant NDV3GM122, which was confirmed by sequencing.

2.7 Substitution of the *F/HN* gene region in the full-length cDNA clone

RNA was extracted from the allantoic fluid of NDV ZJI-inoculated eggs, and reverse-transcription and PCR were performed. The PCR reaction system was prepared as follows: SW 36.5 μ L, 10 \times reaction buffer 5 μ L, dNTPs (10 mmol / L) 1 μ L, HNMF1/HNMF2 (25 pmol / μ L) 1 μ L,

HNMR1/HNMR2 (25 pmol / L) 1 μL, cDNA 5 μL, and Expand High Fidelity Polymerase (3.5 U / μL) 0.5 μL. PCR reaction was programmed as follows: 94°C 4 min, 94°C 35 sec, 48°C 45 sec, 72°C 2.5 min, 5 cycles; 94°C 35 sec, 52°C 45 sec, 72°C 2.5 min, 12 cycles, more than 5 sec was added in the last cycle; 72°C extension 10 min and then 4°C 10 min. The PCR product was purified by gel electrophoresis and ligated with pCR2.1 vector. The positive recombinants were identified by enzyme digestion and named pNDV-FS and pNDV-HNS, respectively. The recombinants were then digested with *BsmBI* and *KpnI*. After the recovery of fragments of interest, ligation, transformation and identification, the positive clones were named pNDV-FHNS. Recombinant plasmids pNDV-FHNS and NDV3GM were digested with *PsiI* and *FspAI*. The digested NDV3GM was dephosphorylated as described above. Finally, similar cloning procedures were performed and the selected positive recombinants were named NDV3GM122, which were also identified by sequencing.

3 Results

3.1 Introduction of mutant nucleotides

Plasmid TNDV4 was digested with *NdeI*, and the 6.5-kb fragment was recovered and self-ligated. The ligation was identified to be successful by digestion with *SacI* and *BamHI*. The three additional nonviral G nts were inserted into the expected position of the plasmid TVTNDV6.5 by mutagenesis. The sequencing result showed that the three G nts were successfully inserted. The recombinant was named TVTNDV6.5PM (Figs. 1 to 3).

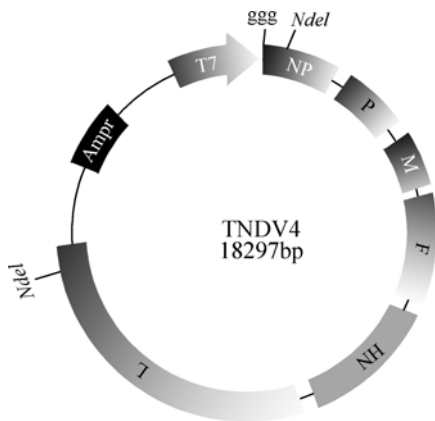


Fig. 1 Schematic representation of G nts insertion

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          10          20          30          40          50
GGGGATCTCG ATCCC GCGAA ATTAATACGA CTCACTATA GGGACCAAACA
GAGAACTCTG GAGGTACGAT AAAAGGCGAA GAAGCAATCG AGATCGTACG
GGTAGAAGGT GTGAACCCCG AGCGCGAGGC CGAAGCTCGA ACCTGAGGGA
ACCTTCTACC GATATGTCGT CTGTTTTTCGA CGAATACGAG CAGCTCCTTG
CTGCTCAGAC CCGCCCTAAC GGAACTCATG GAGGGGGAGA GAAAGGGAGC
ACTTTAAAG TTAGAGTCCC AGTATTTACC CTAAACAGTG ATGATCCGGA
AGATAGATGG AATTTTGC GG TATTCTGTCT TCGGATTACT GTTAGCGAGG
    
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Fig. 4 Sequencing result of the genetic tag of the rescued virus

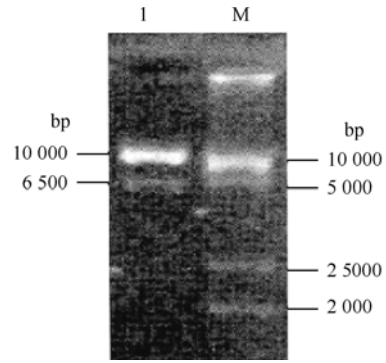


Fig. 2 TNDV4 digestion with *NdeI*

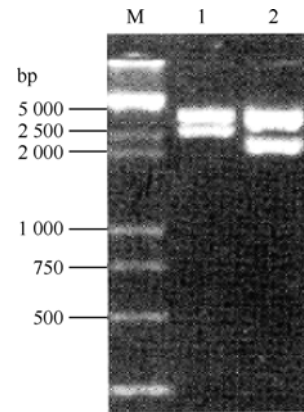


Fig. 3 Identification of TVTNDV6.5P by digestion

3.2 Ligation of full-length cDNA clone

Plasmids TVTNDV6.5PM and TNDV4 were digested with *NdeI* and the fragments of interest were recovered and ligated. The sequencing results indicated that the three G nts were successfully inserted in the expected position of the full-length cDNA clone (Figs. 4 to 6).

3.3 PCR amplification and cloning

The gel electrophoresis result showed that the two 2.1-kb fragments were both successfully amplified. The fragments were ligated with pCR2.1 and identified; the positive recombinants were named pNDV-FS and pNDV-HNS.

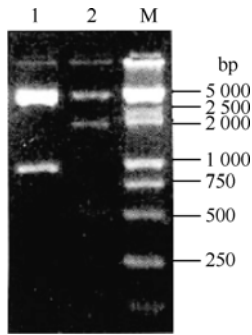


Fig. 5 Identification of pNDV-FS and pNDV-HNS

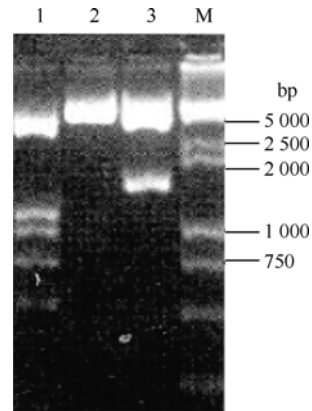


Fig. 8 Identification of recombinant pNDV-FHNS by digestion

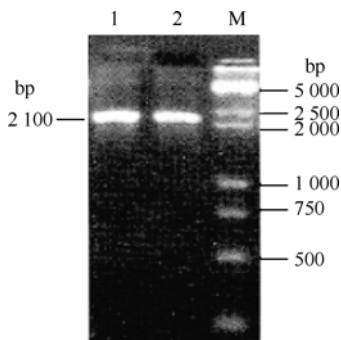


Fig. 6 PCR amplification of the two fragments for substitution

3.4 Ligation and subcloning

Plasmids pNDV-FS and pNDV-HNS were digested with *Bsm*BI and *Kpn*I, respectively. The fragment derived from the digested pNDV-HNS was ligated with the linearized pNDV-FS. The digestion result demonstrated that the two fragments were ligated to each other in the vector pCR2.1 in the right direction (Figs. 7 and 8). The positive clone was named NDV3GM122. The sequencing result showed that the mutant nts in NDV3GM were successfully corrected.

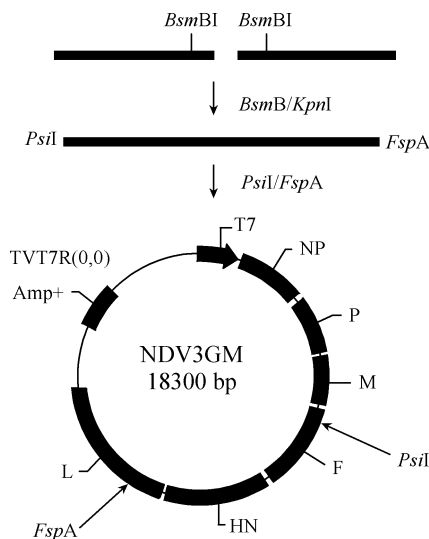


Fig. 7 Substitution of F/HN gene region

3 Discussion and analysis

The epidemics of NDV in goose flocks in some regions of China since 1997 have attracted much attention. We found in our previous research that one NDV strain, ZJI, comprised 15,192 nts in its genomic sequence, and is six nts longer than the published full-length genome of the NDV strains LaSota, Beaudette C and Hitchner B1. The six nts insertion is located in the noncoding region of the *NP* gene between nt 1,647 and nt 1,648 of NDV ZJI (Huang et al., 2004). Is there any relationship between the six nts motif and the amplification, infection, pathogenicity and virulence?

To address this issue, it is not desirable to use conventional methods. On the contrary, it is possible to elucidate the related questions by reverse genetics, which is a powerful and new technology in molecular virology in recent years, especially in the field of functional research (Neumann et al., 2002; Peeters et al., 1999).

Initially, we aimed to establish a reverse genetics system for NDV in our laboratory. We thus completely sequenced the genome of an isolate (ZJI) of NDV obtained after an outbreak in geese (Huang et al., 2004). Meanwhile, the full-length cDNA clone and three helper plasmids *NP*, *P* and *L* of ZJI were established (Liu et al, 2004). We cotransfected the four recombinants into competent cells to rescue the virus but failed although we tried many times. The function of the three helper plasmids was detected by using a constructed minigenome containing the *GFP* reporter gene. The result showed that the *GFP* reporter gene was expressed indicating that all the helper plasmids were functional. Therefore, we can deduce that probably the failure of the rescue was due to the inability of transcription of the full-length cDNA clone or that the functional protein could not be expressed. For this reason, the full-length clone was modified in the present study.

It has been shown previously that the presence of extra G residues in the 5' end of the NDV genomic cDNA clone did not inhibit but increased the rescue of infectious virus from cDNA (Roemer-Oberdoerfer et al., 1999; Peeters et al.,

1999). However, the full-length cDNA clone TNDV4 initially obtained did not have any extra residue in this position. This might influence the function of the transcription clone, and therefore the infectious virus cannot be generated. Thus, in this study, three G nts were introduced into one pair of complementary primers spanning the T7 promoter and the leader sequence of NDV ZJI. PCR mutagenesis was then performed. It is not feasible to perform mutagenesis on the full-length cDNA directly because the cDNA clone is relatively too large. Otherwise, it is most likely to produce mutations during amplification. In addition, the overlap PCR is not suitable for this process because it is difficult to perform overlapping in a 6.5-kb plasmid. For this reason, we first digested the full-length cDNA clone to make it smaller. We then performed site-directed mutagenesis in the digested plasmid to insert the three G nts. Thereafter, the mutated plasmid containing the additional G nts was cloned into the corresponding regions in the cDNA clone. PfuUltra HF DNA polymerase is a proofreading polymerase with high fidelity, which therefore decreases the possibility of mutation during amplification. Additionally, only one pair of primer is used in the mutagenesis.

There is a mutation of 12 nts in the coding region of the *F* gene and 33 nts in the *HN* gene, which was found after comparing the constructed full-length cDNA with the published sequence of NDV ZJI, resulting in a six amino acid change in the *F* and *HN* genes. In order to rectify the mutation, two fragments spanning the whole mutation region were amplified by PCR and then the incorrect sequence was substituted with the correct sequence. The two fragments were ligated by the shared restriction enzyme *Bsm*BI, which was introduced into the reverse primer of the upstream fragment and the forward primer of the downstream fragment. The ligated fragment was digested with *Psi*I and *Fsp*AI, and subcloned into TVTNDV6.5PM, which was digested with the same enzymes.

In PCR amplification, the number of cycles is an important parameter determining the fidelity of the amplified fragment. It was reported by Peeters et al (1999) that the PCR amplification of genomic cDNAs was reduced to only 16 cycles. Thus, in this study, only 17 PCR cycles were used for amplifying the genomic cDNA of NDV ZJI besides using Expand High Fidelity proofreading polymerase. Additionally, the concentration of the template was relatively high. Thus, using these conditions mentioned above resulted in the amplification of the correct sequence.

In the present study, the full-length clone of NDV ZJI

was substituted and modified. The next objective is to rescue the infectious NDV strain ZJI by using the constructed full-length cDNA recombinants and to establish the reverse genetics system of ZJI for future research.

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