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Complete genome sequencing and analysis of an anti-tumor Newcastle disease virus strain

Kaiyang Liu [a,*], Yuanyuan Ma [a], Jing Wang [a], Hongguang Mu [b], Cui Gao [a], Cuijun Yang [a], Jinjun Liu [a]

[a] Life Science Research Center, Hebei North University, Zhangjiakou City, Hebei Province, PR China
[b] Xuangang Hospital, Xuanhua District, Zhangjiakou City, Hebei Province, PR China

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ABSTRACT

HBNU/LSRC/F3, a Newcastle disease virus (NDV) strain stored in our lab, exhibited an anti-tumor ability in our previous studies. Nonetheless, very little is known about its genome sequence, which is vital for further study. Here, the complete HBNU/LSRC/F3 genome was fully sequenced and compared with other NDV sequences. Its genome contained 15,192 nucleotides (nt) consisting of two termini and six genes in the following order: 3′-Le-NP-P-M-F-HN-L-Tr-5′. Phylogenetic analysis indicated that this NDV strain belonged to the Class II genotype IX group. A multibasic amino acid (aa) sequence was found at the cleavage site (^{112}RRQRR↓F^{117}) within the fusion (F) protein, and a 6 nt insertion was present in the 5′ non-coding region of the NP gene. The whole genome sequence was highly similar to other genotype IX NDV genomes reported in China. Overall, this study provides insight into the sequence characteristics of genotype IX NDVs, which will be useful for subsequent investigations.

1. Introduction

Newcastle disease (ND) is a highly contagious disease affecting poultry and wild avian species that occurs upon infection with the Newcastle disease virus (NDV). NDV is the only member of the avian paramyxovirus-1 (APMV-1) species, which belongs to the genus *Avulavirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, and order *Mononegaviriales* (de Leeuw and Peeters, 1999). NDV is an enveloped virus possessing a single-stranded, negative-sense, non-segmented RNA whose genome is approximately 15 kb in length, and its replication is strictly dependent on the "rule of six" (Peeters et al., 2000). The NDV genome contains six open reading frames (ORFs) encoding six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large protein (L) (Dortmans et al., 2011a). In addition, two nonstructural proteins (V and W) can be generated from an mRNA-editing process in which one (V) or two (W) G residues are inserted at a specific position within the P-gene mRNA (Peeters et al., 2004).

NDV strain classification has varied based on the criteria used for classification. NDV strains were categorized as highly virulent (velogenic), intermediately virulent (mesogenic), or non-virulent (lentogenic) based on the level of viral toxicity to embryonating eggs or chickens (Alexander, 1997). Monoclonal antibodies (mAbs) were also used to sort NDV strains into different antigenic groups (Alexander et al., 1997); this classification method, however, failed to distinguish strains with differences in gene sequences that did not affect antibody recognition of the virus (Aldous et al., 2003). Thus, a more recent classification based on variations in genome sequences was employed, leading to the creation of NDV genotypes; this classification was initially based on restriction fragment length polymorphisms and later on the nucleotide (nt) sequence alignment of the F gene, where the latter was more commonly used (Maminiaina et al., 2010; Tirumurugaan et al., 2011; Weingartl et al., 2003).

NDVs are classified into two distinct classes (Class I and Class II), and their genomes are defined into three different genome lengths: 15,186 nt, 15,192 nt and 15,198 nt (Czeglédi et al., 2006). Class I NDVs are 15,198 nt in length and consist of nine genotypes, and all but one are avirulent in chicken (Alexander et al., 1992; Miller et al., 2010). Class II NDVs include eleven genotypes and are more virulent than those of Class I. Genotypes I–IV (15,186 nt) and IX (15,192 nt) emerged before 1960, while genotypes V–VIII (15,192 nt), X, and XI

Abbreviations: aa, amino acid(s); APMV-1, avian paramyxovirus-1; BH3, Bcl-2 homology domain 3; BGI, Beijing Genomics Institute; BSL-2, biosafety level 2; cDNA, DNA complementary to RNA; F, fusion protein; F, phenylalanine; G, guanine; GE, gene end; GS, gene start; HA, hemagglutination; HN, hemagglutinin-neuraminidase; HR, heptad repeat; ICPI, intracerebral pathogenicity index; IFN, interferon; IGS, intergenic sequence; IVPI, intravenous pathogenicity index; kb, kilobase(s) or 1000 bp; L, large protein; L, leucine; M, matrix protein; mAbs, monoclonal antibodies; MDT, mean death time; ND, Newcastle disease; NDV, Newcastle disease virus; NLS, nuclear localization signal; NP, nucleoprotein; Nt, nucleotide(s); ORF(s), open reading frame(s); P, phosphoprotein; pI, isoelectric point; RACE, rapid amplification of cDNA ends; SPF, specific pathogen-free; STAT1, signal transducers and activators of transcription1; SV5, simian virus 5; UTR(s), untranslated region(s); VSV, vesicular stomatitis virus.

* Corresponding author at: Life Science Research Center, Hebei North University, No.11 Zuanshinan Road, Zhangjiakou City 075000, Hebei Province, PR China. Tel./fax: +86 313 4029350.
E-mail address: kaiyang1999@126.com (K. Liu).

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(uncertain) were detected after 1960 (Maminiaina et al., 2010; Miller et al., 2010; Tsai et al., 2004). Interestingly, a novel NDV strain recently found in West Africa was classified under genotype VII of Class II, but harbored a Class I-like 15,198 nt genome due to a 6 nt insertion in the HN–L intergenic region (Kim et al., 2012a). Genotype IX NDVs were found only in China (X.F. Liu et al., 2003). Since the first genotype IX NDV strain, F48E8, was found in the 1940s, several other genotype IX strains were also detected in the 1980s, 1990s, and 2000s (X.F. Liu et al., 2003; Qiu et al., 2011). While genotype IX NDV strains are unique to China, they are quite rare.

For many years, strong evidence has supported a role for NDV in specifically and potently killing a variety of tumors. However, the oncolytic efficiency differs among the NDV strains, which may closely relate to sequence differences among the genomes. Furthermore, the sequence similarity between NDV proteins and cellular apoptotic proteins was recently linked to the ability of NDV to induce tumor-cell apoptosis (Molouki et al., 2011). Many strategies have also been employed to improve the therapeutic potential of NDV; for example, recombinant NDVs containing mutations and exogenous genes have been generated by reverse genetic engineering methods. Thus, determining the genome sequence of NDV strains is important to understand and possibly enhance the anti-tumor function. The anti-tumor activity of the HBNU/LSRC/F3 strain stored in our lab has been previously observed (Liu et al., 2008; Wang et al., 2011). In this study, we have sequenced the complete HBNU/LSRC/F3 genome to provide the molecular basis for subsequent anti-tumor research.

2. Materials and methods

2.1. Virus proliferation

HBNU/LSRC/F3 was stored in our biosafety level 2 (BSL-2) lab (K.Y. Liu et al., 2003). A 0.2 mL aliquot of allantoic fluid containing NDV was separately inoculated into the allantoic cavity of 9-day old, specific pathogen-free (SPF) embryonated hen eggs for virus proliferation. After inoculation at 37 °C for 3 days, the allantoic fluid was harvested and clarified by centrifugation at 850 ×*g* for 45 min at 4 °C. The supernatant was collected, filtered, and tested by hemagglutination (HA) assays. Positive samples showing high HA titers were divided into working stocks and stored at −80 °C until use.

2.2. Viral RNA extraction and RT-PCR

Viral RNA was extracted from 200 μL of the allantoic fluid from HA-positive samples with TIANamp Virus RNA kit (TianGen Biotech Co., Beijing, China) according to the manufacturer's instructions. Viral RNA was then subjected to RT-PCR. A set of 16 primer pairs covering the entire genome was designed based on the GD09-2, F48E8, ZJ/1/86/Ch, FJ/1/85/Ch, JS/1/97/Ch, and JS/1/02/Du genome sequences (GenBank accession numbers HQ317394 and FJ436302–FJ436306, respectively). These primers were 18–21 nt in length and shared similar melting temperatures (between 51 and 55 °C). All primers used for RT-PCR were synthesized by Sangon Biotech Co. (Shanghai, China), and their sequences are displayed in Table 1.

For 12 primer pairs, one-step RT-PCR was successful. Each fragment was amplified in a 56 μL reaction mixture of Quant One Step RT-PCR kit (TianGen Biotech Co., Beijing, China), which contained 12 μL of RNA template and 2.5 U of Platinum® Pfx DNA polymerase (Invitrogen, Beijing, China). The reaction procedure was performed as follows: RT (60 °C for 1 min, 42 °C for 10 min, and 50 °C for 30 min) and PCR (95 °C for 15 min followed by 40 cycles of 94 °C for 30 s, 51–55 °C for 30 s, 72 °C for 60–90 s, and a final extension step at 72 °C for 7–10 min). For the 4 primer pairs for which the above protocol failed, fragments were amplified using two-step RT-PCR. RNA template (8 μL) was denatured (65 °C for 5 min) and added into the 20 μL RT-kit reaction mixture (TianGen Biotech Co., Beijing, China) using the specific sense primer. This mix was incubated at 37 °C for 60 min, and the synthesized cDNA was kept at −20 °C until use. PCR mix (50 μL) was established according to the Platinum® Pfx DNA polymerase instruction manual (Invitrogen Beijing, China), and the reaction proceeded similar to the PCR procedure detailed above for the one-step RT-PCR program.

Table 1
All primers used in this study.

Name	Primer sequence (5′–3′)	Genomic position
1S[a]	AAAGTGAAGGAGCAATCG	31–48
1A[b]	CGGTGTTGATTCCATACTT	935–953
2S	CGGATGAGACAGCAGATG	666–683
2A	AGCGTCTGTAAAGGTGGC	1896–1913
3S	ACATCAACAATCCACACGG	1768–1786
3A	TACGGCACTGAATGAACG	3108–3125
4S	CGTTGAGTAAACCTGCCAC	2899–2917
4A	GCTTCGCCTCTTATCTACAGT	4025–4045
5S	GTGGTGCCGAAGAAGGAT	3833–3850
5A	CAAGTAGGTGGCACGCAT	5441–5458
6S	TACCTCTGATGCTGACCG	4581–4598
6A	GCGTGAGTGCCTATCTATT	5803–5821
7S	ATGCGTGCCACCTACTTG	5441–5458
7A	GCCAATGGAGACTCAAGG	6684–6701
8S	ACACCATCCGTTCTACCG	6376–6393
8A	GGCGGGAGAGAAGTATGAT	7671–7689
9S	CGATGGTTCCCAGTCTATG	7351–7369
9A	GCATTCGTCAGGAAGTGGT	8500–8518
10S	TGCGTCTTGCCAATAGTC	8294–8311
10A	CGCTTCTTTCTGTATCCAT	9591–9609
11S	CGCAGTTACTCTAATCGGAC	9340–9359
11A	CGCATCTTACCTCTCTCG	10,659–10,676
12S	CGTTCAATCCTCCAAGTG	10,449–10,466
12A	CATAATCTGCCAGCGTAAG	11,726–11,744
13S	CGCAGAGGATAATGAGGC	11,422–11,439
13A	TACGAATAGGCGGACCAC	12,764–12,781
14S	CGTGTGGGAAGTTGATTG	12,642–12,659
14A	CGCTGCTTTATCTGAGGT	13,958–13,975
15S	CGTCCCATCTTCTTTCTG	13,656–13,673
15A	ATTATCCCTTGACCGCAT	14,964–14,981
16S	ATGACTCAGATGACCCAGAT	14,543–14,562
16A	GCTTGGTGAATGACAGAACT	15,162–15,181
3LA[c]	GAGAGATATGAGAGCACCTTGTCTGAGT	322–349
3SA	CGGGCAGAATACCGCAAAGTTCCATC	264–289
5LS	GTCCATTCTGTGCAGAGAGTTTAGTGAG	14,502–14,529
5SS	CAATACTGGGTCTCAGAGTCAAAAATC	14,724–14,750
CL+[d]	CGCCAGGGTTTTCCCAGTCACGAC	–
CL−	GTCGTGACTGGGAAAACCCTGGCG	–

[a] "S" stands for forward primer.
[b] "A" stands for reverse primer.
[c] These primers (3LA, 5LS, 5SS, CL+ and CL−) were designed as previously reported (Qiu et al., 2011).
[d] CL+ and CL− are anchor primers.

2.3. Amplification of the 3′ and 5′ end of the viral genome

The 3′- and 5′-end sequences of the viral genome were amplified by rapid amplification of cDNA ends (RACE). For 3′-RACE, the 3′ end of genomic RNA was ligated to the 5′ end of phosphated adaptor primer CL+ using T4 RNA ligase (Fermentas, Beijing, China). The ligation mix was prepared as previously reported (Qiu et al., 2011) and incubated at 10 °C for 18 h, which was followed by enzyme denaturation (75 °C for 15 min) and denaturation of the resulting CL+-ligated RNA (65 °C for 5 min). Then, 8 μL of the ligated product was used to make cDNA with the anti-adaptor primer CL−, which was complementary to the CL+ adaptor primer. The reaction was performed with Quant RTase (TianGen Biotech Co., Beijing, China) according to the manufacturer's instructions. PCR amplification was performed using CL− as the sense primer and 3LA and 3SA as the antisense primers, which were specific for the viral NP gene. For 5′-RACE, cDNA was first produced by Quant RTase (TianGen Biotech Co., Beijing, China) with the specific 5LS primer. The subsequent steps, including removing residual RNA, denaturing