Contribution of the length of the HN protein and the sequence of the F protein cleavage site to Newcastle disease virus pathogenicity

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Newcastle disease virus (NDV) possesses two envelope spike glycoproteins: the haemagglutinin–neuraminidase (HN) protein and the fusion (F) protein. The HN protein, which is responsible for virus attachment to sialic acid-containing receptors, varies in length due to differences in the sizes of the ORFs. An HN protein precursor of 616 aa has been found in avirulent but not in virulent NDV strains, whereas an HN protein of 571 aa can be detected in highly virulent strains only. An HN protein of 577 aa is present in virulent and avirulent strains. The F protein, which mediates virus–cell fusion, requires proteolytic activation at an internal cleavage site, whose amino acid composition determines cleavability by various proteases. Here, the functional significance of the length of the HN protein in combination with F protein cleavage sites typical for virulent (velogenic and mesogenic) or avirulent (lentogenic) strains was investigated. To this end, site-directed mutagenesis was used to construct recombinant NDV on the basis of an infectious clone of the lentogenic vaccine virus Clone-30. Only recombinant NDV expressing an F protein with a multibasic cleavage site typical of virulent strains was able to spread efficiently in cell culture, irrespective of the size of the HN protein. Moreover, as determined by the intracerebral pathogenicity index (ICPI) in 1-day-old, specific-pathogen-free chickens, pathogenicity was influenced by the cleavability of the F protein and not by the length of the HN protein. The maximum ICPI value obtained for these recombinants was 1.3, as compared to a possible maximum of 2. This demonstrates that the modifications introduced did not result in the conversion of the lentogenic Clone-30 to a velogenic strain with an ICPI value of >1.5 and suggests the involvement of additional virulence determinants that contribute to the pathogenicity of NDV.

INTRODUCTION

Newcastle disease virus (NDV) is a member of the new genus Avulavirus within the family Paramyxoviridae (Mayo, 2002a, b). The NDV nonsegmented, negative-sense, single-stranded RNA genome contains six genes in the order 3′-NP-M-F-HN-L-5′, which encode the six major structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin–neuraminidase (HN) and the large (L) RNA-dependent RNA polymerase (Lamb & Kolakofsky, 2001). Like other members of the subfamily Paramyxovirinae, NDV edits its P gene and transcribes three P gene-derived mRNA species, encoding the P, V and W proteins (Steward et al., 1993). The genome size of 15 186 nt (de Leeuw & Peeters, 1999; Römer-Oberdörfer et al., 1999; Krishnamurthy & Samal, 1998) is a multiple of six, which is a characteristic of most members of the Paramyxovirinae (Kolakofsky et al., 1998). Studies with NDV minigenomes showed that replication of NDV was indeed dependent on the rule-of-six (Peeters et al., 2000).

Newcastle disease is a highly contagious disease of many avian species, which leads to substantial economic losses in the poultry industry. The severity of disease depends on the virus strain and the host species. NDV strains can be differentiated on the basis of their pathogenicity for chickens into strains of high (velogenic), intermediate (mesogenic) or low (lentogenic) virulence. Velogenic NDV strains cause acute infections with high mortality, preceded by respiratory and neurological signs or haemorrhagic lesions in the gut, whereas mesogenic strains cause respiratory disease in young birds and decreased egg production in laying flocks. In contrast, lentogenic strains may produce only mild respiratory signs in young chickens. The pathogenicity of a given NDV isolate can be determined under laboratory conditions by assessing the intracerebral pathogenicity index (ICPI) in 1-day-old, specific-pathogen-free (SPF) chickens. Accordingly, NDV isolates with an ICPI value of <0.5 are classified as lentogenic, whereas isolates with ICPI values of 0.5–1.5 and 1.5–2.0 are categorized as
mesogenic and velogenic strains, respectively (Alexander, 1998). Lentogenic strains are preferentially used for vaccine preparations due to their low pathogenicity for chickens. Vaccination against Newcastle disease is widely practiced using inactivated as well as live vaccines.

The envelope of NDV virions contains two transmembrane glycoproteins, the virus attachment protein, HN, and the fusion protein, F, which form spike-like protrusions on the outer surface of the virion. Both are important to initiate infection. The HN protein is responsible for the attachment of virus particles to sialic acid-containing receptors of the host cell. It is the largest glycoprotein molecule and carries both haemagglutinating and neuraminidase activities (Scheid & Choppin, 1973; reviewed by Morrison & Portner, 1991). Comparison of the nucleotide sequence of the HN genes of 13 NDV isolates demonstrated three different HN genotypes according to differences in the position of the stop codon of the HN ORF. Consequently, protein products of 616, 577 or 571 aa could be translated (Sakaguchi et al., 1989). The HN protein of 616 aa (Sato et al., 1987) was detected as a precursor protein, HN0 (Nagai et al., 1976; Nagai & Klenk, 1977), which is converted into the biologically active glycoprotein HN by proteolytic removal of a small glycosylated fragment (Garten et al., 1980). Depending on the protease involved, different carboxy termini of HN may be created, as shown for the avirulent strain Ulster (Schuy et al., 1984). Most NDV strains, including lentogenic, mesogenic and velogenic strains, exhibit HN proteins of 577 or 571 aa, since termination codons are located before the HN0 stop codon. In these proteins, posttranslational cleavage is not required for functionality (Alexander, 1997). It is of interest to note that, to date, only the shortest HN protein (571 aa) has been found in velogenic strains. The carboxy-terminal extension of the HN protein of avirulent strains (Queensland, Ulster and D26) appears to be a structural feature responsible for the functional impairment of the HN protein precursor of these strains (Gorman et al., 1988, 1992).

The F protein mediates fusion of the virion envelope with the cellular plasma membrane (Nagai et al., 1989). It is synthesized as a precursor protein, F0, and is activated by proteolytic cleavage between aa 116 and 117. The resulting subunits, F1 and F2, remain linked by disulfide bonds. The fusion-competent cleaved form of the F protein is not essential for formation of virus particles but is required for infectivity (Nagai & Klenk, 1977). The F proteins of pathogenic (velogenic and mesogenic) strains are cleaved in a wide range of different host cells because their amino acid sequence at the cleavage site is a substrate for proteases of the furin family (Gotoh et al., 1992; reviewed by Nagai, 1993). In contrast, the F proteins of lentogenic strains exhibit a different amino acid motif at the cleavage site and are susceptible to proteolytic cleavage only in a restricted number of cell types (Nagai et al., 1979). They are cleaved by extracellular enzymes, such as a protease released by Clara cells in the respiratory tract or a protease with factor X-like activity in the allantoic fluid of embryonated eggs (Gotoh et al., 1990; Kido et al., 1992; reviewed by Nagai, 1993). Cleavage of these F proteins in tissue culture depends upon the addition of exogenous trypsin (Nagai & Klenk, 1977). It has been shown that a cluster of basic amino acid residues at the F protein cleavage site, as found in pathogenic NDV strains, is a prerequisite for the pathogenic phenotype (Glickman et al., 1988; Nagai et al., 1976; Toyoda et al., 1987).

Recently, the generation of recombinant NDV by reverse genetics has been described (Krishnamurthy et al., 2000; Peeters et al., 1999; Römer-Oberdörfer et al., 1999). By applying this system, it is now possible to perform targeted mutagenesis of the viral genome to investigate the effects of distinct mutations on the phenotype of the virus. In this study, the F protein cleavage site and/or the position of the stop codon in the HN ORF was altered by site-directed mutagenesis in a full-length cDNA clone of the lentogenic strain Clone-30. The recombinant virus mutants obtained were analysed in vitro and in vivo.

**METHODS**

**Viruses and cells.** Recombinant viruses were generated from a full-length cDNA copy of the lentogenic NDV vaccine strain Clone-30 (rNDV), as described previously (Römer-Oberdörfer et al., 1999). BSR-T7/5 cells, which stably express phage T7 RNA polymerase (Buchholz et al., 1999), were used for recovery of infectious virus.

**Introduction of mutations into full-length NDV cDNA.** The full-length cDNA clone, pFL-NDV-1, expressing the antigenome RNA of Clone-30 (Römer-Oberdörfer et al., 1999) was used to introduce various mutations, as shown in Figs 1 and 2. Introductions of F and HN sequence alterations were done using the USE Mutagenesis kit (Amersham Pharmacia). Mutagenesis of the F cleavage site to generate rNDVF1 was done as described previously (Mebatsion et al., 2001). To alter the position of the HN stop codon, PCR was performed on pFL-NDV-1 using the forward primer P3F and the reverse primer P3R (Table 1). The PCR fragment was introduced into the Smal site of pUC18 for site-directed mutagenesis. The Scal/Mid selection primer and the mutagenic primer MPHNI were used to generate HN with 571 aa and primers MPHN2 and MPHN2a were used to generate HN with 616 aa. In a second mutagenesis reaction, MPHN2U was used to create the extension of the HN ORF in correspondence to the sequence of NDV strain Ulster (Table 1). Plasmids containing the desired alterations were then digested with SpeI/BsaI and the resulting 759 bp mutant fragments were exchanged with the corresponding fragment of the plasmid pFL-NDV-1. F protein cleavage site alterations were combined with the HN proteins of different lengths using the NotI sites of pFL-NDV-1 (Römer-Oberdörfer et al., 1999).

**Transfection and virus recovery.** Transfection experiments were done as described previously (Römer-Oberdörfer et al., 1999). For recovery of the new recombinant viruses, the full-length antigenome-containing plasmids pFL-NDV-2, -5, -6, -7 or -8 (Figs 1 and 2) were used. Supernatants and cell monolayers were harvested 72 h after transfection and inoculated into the allantoic cavity of 10-day-old, embryonated, SPF chickens' eggs. A rapid-plate haemagglutination (HA) test (CEC, 1992) using harvested allantoic fluid was carried out after 4–5 days of incubation to confirm the presence of virus.
RT-PCR and sequencing. RNA was prepared from 100 μl allantoic fluid using the Purescript RNA Isolation kit (Biozym). RT-PCR was performed using primers described previously for MluI sites in the noncoding regions of the NP and L genes (Römer-Oberdörfer et al., 1999). For amplification of a fragment including the altered F cleavage site, primers P11F and P11R (Table 1) were used. Amplification of a fragment including the alterations of HN was performed with primers P12F and P12R (Table 1). The resulting PCR fragments were sequenced directly with specific primers using an automated sequencer (LiCor, MWG Biotech) and sequences were analysed using the Wisconsin package, version 10.0 (GCG).

Indirect immunofluorescence assay (IFA). Infected cells were fixed with acetone or 3% paraformaldehyde. Detection of NDV was done by IFA using monoclonal antibody (mAb) HN-10 (Werner et al., 1999) directed against the NDV HN protein, and FITC-conjugated goat anti-mouse IgG Fab2 (Dako) or anti-NDV FITC-conjugate (Gamakon, Bioveta).

Determination of ICPI values. ICPI values of the different recombinant NDV isolates were determined following European guidelines (CEC, 1992) in 1-day-old SPF chickens. The appearance of clinical signs and mortality was scored for 8 days, as described by Alexander (1998).

Virus growth. QM7 (quail muscle, clone 7) cells were infected with recombinant NDV at different m.o.i. and incubated at 37 °C in MEM supplemented with 10% FCS in a 3% CO2 atmosphere. Cells with supernatants were frozen at the times indicated (Fig. 3a). Tittrations were performed in duplicate. Briefly, 100 μl of tenfold serial dilutions in MEM were added to 10^5 cells per well. After 18 h of incubation, cells were fixed with 3% paraformaldehyde and virus titres (TCID50) were determined by IFA using anti-NDV FITC-conjugate. Since all recombinant viruses with an F cleavage site with single basic amino acid residues were unable to multiply in permanent cell cultures, 200 μl of a virus dilution containing 10^6 TCID50 ml^-1 was inoculated into the allantoic cavity of 10-day-old, embryonated chickens' eggs. At the times indicated, the allantoic fluid of inoculated eggs containing live embryos was harvested and

**Fig. 1.** Alteration of amino acids at the F protein cleavage site. Recombinant NDV (rNDV) isolates were recovered from full-length antigenome-containing plasmids (pfl-NDV-1, -2, -5, -6, -7 or -8). Respective amino acid sequences at the F protein cleavage sites are shown. Sequences from naturally existing strains (lower six viruses) are provided for comparison (Toyoda et al., 1989). Differences to Clone-30 are shown in bold.

**Fig. 2.** Alteration of the position of the HN protein stop codon by site-directed mutagenesis. For comparison, homologous regions of naturally occurring virus strains (lower six viruses) (Sakaguchi et al., 1989) are given. Sequence differences to Clone-30 are shown in bold and amino acids at the carboxy terminus of the HN protein are boxed.
clarified by centrifugation for 10 min at 925 g. Supernatants were pooled, frozen and thawed, and, thereafter, used for titration in tissue culture, as described above.

**Radiolabelling, cell lyses and immunoprecipitation.** Primary chicken embryo fibroblasts (CEF) \((1 \times 10^6)\) were infected with recombinant NDV at an m.o.i. of 1. After 3 h of infection, the inoculum was replaced with DMEM without methionine and cysteine (ICN) and the cells were incubated for a further 60 min at 37 °C. Subsequently, cells were radiolabelled with 50 μCi \([^{35}S]methionine\) (Trans-Label, ICN) for 16 h at 37 °C.

After labelling, cells were washed twice with PBS and lysed with 0.5 ml RIPA buffer \((10 \text{ mM sodium phosphate}, 10 \text{ mM EDTA}, 1 \% \text{Triton X-100}, 1 \% \text{sodium deoxycholate}, 1 \% \text{SDS} \text{ and } 40 \text{ mM sodium fluoride})\) and stored for at least 2 h at \(-20^\circ\text{C}\). After thawing, lysates were clarified by centrifugation at 20,000 g for 20 min and subsequently incubated with antisera for 1 h at 4 °C. For precipitation, prewashed Pansorbin cells (Calbiochem) were added and the mixture was incubated in RIPA buffer for 1 h at 4 °C. Immunoprecipitates were washed twice with RIPA wash buffer \((10 \text{ mM sodium phosphate}, \text{pH 7.1}, 10 \text{ mM EDTA}, 1 \text{ M NaCl} \text{ and } 0\% \text{ Triton X-100})\) and once with sterile, distilled water. Immunoprecipitates were resuspended in sample buffer, heated to 95 °C for 5 min and centrifuged at 12,000 g for 2 min. Immunoprecipitated proteins were separated by SDS-PAGE under reducing conditions, exposed to a BAS MS 2325 imaging plate (Raytest) and analysed using a fluorescent image analyser (Fujifilm).

**RESULTS**

**Generation of mutant NDV from cDNA**

To investigate the influence of the length of the HN protein and the amino acid sequence at the F protein cleavage site on NDV pathogenesis, the modifications shown in Figs 1 and 2 were introduced into the full-length Clone-30 cDNA plasmid, pfl-NDV-1. Plasmids containing the altered full-length antigenome were cotransfected into BSR-T7/5 cells with three support plasmids expressing the NP, P and L proteins. Supernatants were harvested 72 h after transfection and transfected cells were subjected to immunostaining using the anti-HN mAb. With all full-length cDNAs constructed, cells exhibiting bright, NDV-specific immunofluorescence were detected. For virus propagation, transfection supernatants were inoculated directly into embryonated, SPF chickens’ eggs, as they provide the best substrate for the multiplication of NDV of different pathotypes (Garten et al., 1980; Nagai et al., 1976). After 4–5 days of incubation, allantoic fluids were harvested and used for HA tests. HA titres were detectable after the first egg passage of all transfection supernatants, indicating that all newly generated NDV recombinants are replication competent in embryonated eggs.

The sequence of the F protein cleavage site is an important determinant for NDV pathogenicity

All recombinant viruses with an F protein cleavage site specifying two pairs of basic amino acids at the carboxy terminus of F2 and a phenylalanine at the amino terminus of F1 (rNDVF1, rNDVF1HN1 and rNDVF1HN2U) (Fig. 1)
could be propagated in tissue culture (Fig. 3a). Typically, extensive syncytia were formed, as observed by indirect immunofluorescence (Fig. 4). Moreover, the ICPI value increased from 0.01 for the parental recombinant Clone-30 (rNDV) to 1.28 for rNDVF1 (Table 2). The presence of the correct mutations within the genomes of the recombinant viruses used for ICPI determination was verified by PCR and sequencing (data not shown). These results confirm the importance of the F protein cleavage site for NDV pathogenicity.

The size of the HN protein does not influence NDV pathogenicity

In certain avirulent strains of NDV (e.g. Ulster and D26), the HN precursor HN0 is 616 residues long and a post-translational cleavage is required to generate a biologically active HN protein. In contrast, more virulent NDV strains have termination codons located before the end of the HN0 gene, resulting in HN proteins of 577 or 571 residues. To determine the role of the length of the HN protein in NDV virulence, we generated recombinant viruses with an F cleavage site (GRQGR*L) of lentogenic NDV and with HN lengths of 616 aa (rNDVHN2U) or 571 aa (rNDVHN1). For rNDVHN1, the AGA codon of Clone-30, encoding arginine at position 572, was altered to a stop codon (TAA) to create a 571 aa HN protein. For rNDVHN2U, the stop codon (TAG) at position 578 was changed to a CGG codon, encoding an arginine. Both downstream stop codons (positions 583 and 615) and other different amino acids were also altered (Fig. 2) to enable the translation of a 616 aa HN protein, as in the HN protein of strain Ulster. All three recombinant viruses (rNDV, rNDVHN1 and rNDVHN2U) replicated at a comparable efficiency in embryonated eggs (Fig. 3b), although growth of rNDVHN2U was somewhat delayed but reached nearly the same final titre as the other viruses. As expected, no productive infection could be detected in cultured QM7 cells independent of m.o.i., as shown here for an m.o.i. of 1 (Fig. 3a), not even by IFA (Fig. 4).

Determination of the ICPI values for all three viruses revealed only marginal differences (Table 2). Several rNDVHN2U-infected animals showed temporary mild respiratory symptoms, resulting in an ICPI value of 0.1875. Further examination of these chickens demonstrated a septicemia with Enterococcus and Pediococcus species, which did not occur in control animals. Even though it is

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not clear whether the mild clinical signs were due to the bacteria observed or the virus infection, the recombinant virus could clearly be classified as lentogenic.

We then characterized velogenic versions (F cleavage site, RRQKR*F) of recombinant viruses with HN lengths of 616 aa (rNDVF1HN2U), 577 aa (rNDVF1) or 571 aa (rNDVF1HN1). All three mutants replicated efficiently in QM7 cell culture (Figs 3a and 4) as well as in embryonated eggs (Fig. 3b). The ICPI values determined for rNDVF1, rNDVF1HN1 and rNDVF1HN2U were 1.28, 1.30 and 1.21, respectively (Table 2), indicating that the length of the HN protein has no detectable role in NDV pathogenicity. Interestingly, none of the mutations or combinations of mutations introduced resulted in the generation of recombinant NDV with a highly virulent, velogenic phenotype, with an ICPI of >1.5.

To verify the different length of the HN proteins, immunoprecipitations of recombinant virus-infected cells were analysed under reducing conditions (Fig. 5). Unfortunately, the available mAb against NDV HN did not detect the protein in all of our recombinant viruses. However, the HN protein of a velogenic wild-type isolate (R 5/93) was detected by mAb HN-10, which is shown in Fig. 5 to identify the HN protein in comparison with precipitations using a polyclonal NDV antiserum (aNDV). It is obvious that the HN proteins of the different recombinant NDV differ in apparent molecular mass corresponding to the location of the stop codon, demonstrating the correct expression of the mutated HN proteins.

**DISCUSSION**

In this study, the reverse genetics system described recently for NDV has been used to generate mutant viruses with alterations at the proteolytic cleavage site of the F protein and the carboxy terminus of the HN protein to assess their influence on pathogenicity. For the F protein, it is generally accepted that the number of basic amino acids immediately upstream of the cleavage site determines cleavability of the protein by different proteases and, consequently, the ability of the virus to multiply and spread both in cell culture and in animals. Our data confirm these findings, in that all recombinant viruses containing two pairs of basic amino acids (RRQKR*F) (rNDVF1, rNDVF1HN1 and rNDVF1HN2U) multiply and spread in tissue culture and exhibit an ICPI value of >1.2. Such ICPI values are typical of mesogenic (intermediate virulence) NDV strains. A similar result has been obtained by Peeters et al. (1999), who tested a recombinant NDV with a sequence motif of RRQR*F.

NDV strains with different virulence for chickens also

| Table 2. Pathogenicity of different recombinant NDV |
|-----------------|--------|--------|
| Virus           | ICPI   | Pathotype |
| rNDV            | 0.01   | Lentogenic |
| rNDVF1          | 1.28   | Mesogenic |
| rNDVHN1         | 0.04   | Lentogenic |
| rNDVHN2U        | 0.19   | Lentogenic |
| rNDVF1HN1       | 1.30   | Mesogenic |
| rNDVF1HN2U      | 1.21   | Mesogenic |
exhibit differences in the length of the HN protein ORF, which depends on the location of a stop codon. For example, a 571 aa HN protein is expressed only by velogenic NDV strains (e.g. Miyadera and Herts), whereas avirulent strains (Queensland, Ulster and D26) produce a 616 aa HN0 precursor protein that requires proteolytic cleavage to become a biologically active HN. An intermediate HN of 577 residues is expressed by lentogenic (e.g. Clone-30 and B1) or virulent strains (e.g. Texas and Beaudette C strains). In the HN0 genes, the stop codons at nt 8125–8127 (571 aa) and 8143–8145 (577 aa) are replaced by arginine codons, and translation stops 135 nt downstream. Thus, the full-length HN0 protein is 616 aa long. In our assays, the mutations regarding the length of the HN protein did not alter the virulence of the mutant viruses for 1-day-old chickens after intracerebral inoculation. However, further studies should reveal whether the length of the HN protein plays a role in the ability of the viruses to spread and propagate in various organs of chickens after parenteral administration. Our data show further that by site-directed mutagenesis of the stop codon in the HN protein of Clone-30, an otherwise noncoding region trailing the ORF was converted to become part of a functional protein. The resulting 616 aa HN0 shows a clear increase in apparent molecular mass as compared to the 571 or 577 aa HN proteins (Fig. 5). It is assumed that HN0 is proteolytically processed by a host protease at a single arginine at position 572 or 578, which represent stop codons for the smaller HN proteins. It is also conceivable that the cleavage occurs in a stepwise fashion using other arginines of the HN extension. To confirm this assumption and to delineate the influence of the 45 additional amino acids for the replication process, arginine residues of the extension sequence need to be eliminated or substituted.

NDV strains of different pathogenicity differ in their F protein cleavage site sequence and exhibit HN ORFs that result in primary translation products of different sizes. Our data show that the sequence at the F protein cleavage site is the prime determinant of NDV virulence, irrespective

**Fig. 5.** Immunoprecipitation of NDV proteins from infected CEF. Cells were infected with Clone-30 and six recombinant NDV isolates for 3 h followed by labelling with [35S]methionine for 16 h. After cell lysis, extracts were analysed by immunoprecipitation with a rabbit polyclonal NDV-specific antiserum (aNDV) or mAb HN-10. Precipitates were subjected to SDS-PAGE (10% acrylamide) under reducing conditions. HN proteins are marked by arrows. Apparent molecular masses are indicated in kilodaltons.

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of the length of the HN protein. The highest ICPI value obtained for the recombinants with the virulent sequence of the F protein cleavage site was 1.3, which is indicative of only intermediate pathogenicity. This parallels previous findings by Peeters et al. (1999), who obtained an ICPI value of 1.28 for a recombinant virus with an F protein cleavage site sequence of RRQRR*F. It is of interest to note that by alteration of the F protein cleavage site of a lentogenic strain to the consensus cleavage site of a virulent strain of NDV, it was possible to generate recombinants only with intermediate virulence (mesogenic) but not with high virulence (velogenic). de Leeuw et al. (2003) have shown that pathogenicity of recombinant NDV with an F protein cleavage site similar to F₁ of our recombinants increases from an ICPI value of 1.3 to 1.5 after one passage in chickens’ brains without further alteration of the F protein cleavage site. This suggests that the F protein cleavage site is not the sole determinant of NDV virulence but that other mutations in the NDV genome may cause the increase in pathogenicity. Recently, we have shown that the NDV V protein, which is produced as a result of editing of the P gene mRNA, plays a role in pathogenicity (Mebatsion et al., 2001). It was demonstrated that the level of V protein expression correlates directly with NDV virulence for chicken embryos. Therefore, it is of great interest to investigate whether the frequency of the P gene mRNA editing, and thus the level of the V protein, varies in virulent and nonvirulent strains, thereby contributing to variation in pathogenicity among NDV strains with an identical F protein cleavage site. However, a possible influence of mutations within other proteins, e.g. the polymerase, or of alterations of additional single amino acids in the surface glycoproteins, which may cause more structural changes within these proteins, is conceivable.

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REFERENCES


