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ARTICLE

The Influence of Newcastle Disease Virus Major Proteins on Virulence

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ABSTRACT

The Newcastle disease virus (NDV) negative-strand RNA genome contains six genes. These genes encode nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and RNA-dependent RNA polymerase (L) proteins. The six proteins affect the virulence of NDV in different ways, but available information on the six proteins is disparate and scattered across many databases and sources. A comprehensive overview of the proteins determining NDV virulence is lacking. This review summarizes the virulence of NDV as a complex trait determined by these six different proteins.

1. Introduction

Newcastle disease (ND) is an important poultry disease that causes huge economic loss to the poultry industry and is distributed around the world. ND is caused by Newcastle disease virus (NDV), which infects more than 240 species of birds, and is spread mainly through direct contact between infected birds and healthy birds. ND outbreaks were first reported in Java, Indonesia, and Newcastle upon Tyne, UK, in the mid-1920s. Within a couple of years, ND spread rapidly and became a serious poultry epidemic worldwide.

According to the latest classification issued by the International Committee Taxonomy of Viruses (ICTV), NDV is named *Avian orthoavulavirus 1* (AOAV-1), classified in the genus *Orthoavulavirus*, subfamily *Avulavirinae*, family *Paramyxoviridae*. All NDV strains belong to a single serotype but have multiple genotypes. NDV strains are classified into two classes, I and II, based on genome and fusion gene sequence lengths [1]. Class I viruses contain a single genotype with a genome length of 15,198 nucleotides (nt). Class II consists of 15 genetic groups, I–XV, with genome lengths of either 15,186 or 15,192 nt [2]. The viruses of class I are avirulent in general, while class II contains virulent and avirulent strains [3]. Currently, the predominant strains circulating the world belong to genotypes V, VI, and VII of class II, while

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commonly used vaccines are against LaSota and B1 of class II's genotype II, which were both isolated 60 years ago. These commonly used class II vaccines offer partial protection against current circulating NDV. However, birds immunized with the traditional class II vaccines can still be infected with many currently endemic NDV strains. Live attenuated vaccines or inactivated vaccines developed from currently circulating genotype strains provide improved immune protection against current strains, suggesting the importance of developing vaccines against currently circulating genotypes [4].

The NDV genome includes six open reading frames (ORFs), which encode a nucleoprotein (NP), a phosphoprotein (P), a matrix protein (M), a fusion protein (F), a haemagglutinin-neuraminidase (HN), and an RNA-dependent RNA polymerase (L). A W protein and a V protein are generated by RNA editing during P gene transcription. Viral infection is initiated by adhesion of virions to the surface of host cells. Similar to other paramyxoviruses, the viral HN protein binds to sialic acid receptors on target cell surfaces, triggering the F protein to facilitate fusion of the viral envelope with the plasma membrane of the host cell by a pH-independent mechanism [5]. After entry, the virus replicates, transcripts, and translates in the cytoplasm. Finally, all components of the virion are transported to the plasma membrane and bud with the participation of the M protein [6]. Various steps in the NDV infection cycle require the participation of multiple viral proteins, and its virulence is determined by multiple proteins.

2. NP Protein

The NDV NP gene is 489 amino acids (aa) long and is highly conserved within different virulent strains. NP contains two domains: a highly conserved structural N-terminal region (N_{CORE}) and a C-terminal region (N_{TAIL}). The N-terminus 401 aa share high sequence identities among all NDV isolates, while the C-terminal region is highly variable. The glutamic acid residue at position 402 (E402) in the NP's N_{TAIL} is essential for NDV RNA synthesis [7].

NP binds to a viral RNA-dependent RNA polymerase (RdRp) complex, composed of P and L proteins, and facilitates the formation of the active ribonucleoprotein (RNP) complex, which is necessary for transcription and replication [8]. Moreover, NP regulates viral genome transcription and replication by interacting with P alone or with itself [9]. Zhan *et al.* reported that NDV infection upregulated PI3K/Akt/mTOR and p38 MAPK/Mnk1 pathways to promote translation of viral mRNA. They proposed that during NDV infection, the NP protein is essential for the selective cap-dependent translation of viral mRNA by binding to eIF4E [10].

3. P, V, and W Protein

The NDV polycistronic phosphoprotein (P) gene is approximately 1,450 nucleotides long and encodes a P protein of 395 aa [11]. The P protein plays significant roles in genome replication and transcription. The P protein serine and threonine residues are highly phosphorylated and are considered necessary for virus replication and transcription [12]. In addition, the P protein can interact with the L protein to recruit an NP-RNA template to assemble the RNP complex, which is critical for RNA synthesis [9].

Paramyxovirus P genes encode more than one protein via an RNA editing mechanism. The V and W proteins are expressed through this RNA editing process during NDV infection. In general, the presence or absence of non-templated guanosine nucleosides inserted in NDV P gene-derived transcripts at ORF position 484 determines which protein is encoded. An mRNA without the guanosine nucleoside inserted encodes the P ORF, whereas mRNAs with a +1 or +2 frameshift encode the V or W protein, respectively. The P/V/W proteins all have the same N-terminal regions but different C-terminal regions [13]. Previous studies have shown that the editing frequencies for the P, V, and W mRNAs in NDV-infected cells are 68% for P, 29% for V, and 2% for W [14].

In addition to being a P gene derivation product, the V protein is an antagonist of α/β interferon (IFN-α/β), which contributes to NDV virulence. Host cellular innate immune systems become weakened in stable DF-1 cell lines expressing the V protein [15]. Other studies have shown that the V protein can antagonize IFN and suggest that the NDV V protein functional domain inhibiting IFN is in its C-terminal domain and promotes degradation of the signal transducer and activator of transcription 1 protein (STAT1) immediately after STAT1 is phosphorylated [16]. Others have reported that the V protein can upregulate SOCS3 expression via the MEK/ERK pathway to benefit NDV replication [17]. NDV V proteins target MAVS for ubiquitin-mediated degradation, leading to down-regulated expression of the proteins in the downstream IFNs pathway and the promotion of virus replication [18].

Only in recent years has more W protein research began to emerge [19]. We previously investigated W protein length diversity, demonstrated the presence of the W protein in different NDV strains, and identified its subcellular localization [20]. Our latest results show that the W protein has a nuclear export signal (NES) motif. Due

to a nuclear localization signal (NLS), the W protein can be transported into the nucleus via KPNA family proteins, including KPNA1, KPNA2, and KPNA6. The W protein's NES motif facilitates its return to the cytoplasm in a CRM1-independent manner. After deletion of its NES, the W protein remains in the nucleus and cannot be sent back to the cytoplasm. We also found that the location of the W protein affects virulence [21].

4. M protein

The matrix protein is located in the viral envelope's inner surface, and is considered the third NDV envelope protein. Like other paramyxovirus M proteins, the NDV M protein is a nucleocytoplasmic shuttling protein [22]. In addition to viral particle assembly function in the cytoplasm and budding at the cell membrane, the NDV M protein localizes in host nuclei at an early stage after infection and remains there during infection. This M protein nuclear-nucleolar localization is thought to regulate a balance between viral replication and transcription and inhibit host protein synthesis, similar to that seen in the M protein of other negative-sense RNA viruses, including the M protein of the measles virus and in the *Vesicular stomatitis Indiana virus* (VSV) [23]. These studies support the notion that the NDV M protein is an essential multifunctional viral protein that plays an essential role in virus growth and replication.

Among the M proteins of paramyxoviruses, only Sendai virus (SeV) and NDV M proteins have been observed in the nucleoli. A previous study showed that the NDV M protein enters the nucleus and co-locates with the nucleoli through a bipartite NLS independent of other viral proteins [24].

Most paramyxovirus M proteins, including NDV, are largely hydrophobic, but do not have transmembrane properties. This is consistent with observed peripheral attachments of paramyxovirus M proteins to the viral membrane. However, the basic residue distribution in the NDV M protein sequence is not uniform. M protein charge distribution analysis shows that the N-terminal 100 aa are acidic in general, but the remainder are strongly basic. Recent studies have reported that most cellular or viral proteins possess essential residue-rich peptides to mediate nuclear or nucleolar localization [25].

The M protein can interact with a variety of host proteins. In this way the M protein inhibits host protein function and promotes NDV growth and replication. Host protein interactions with M proteins have been demonstrated using co-immunoprecipitation (Co-IP) and a yeast two-hybrid system [26]. The NDV M protein and avian nucleophosmin B23.1 co-localize in the nucleolus in co-transfected HEK-293T cells. The binding of M protein with B23.1 was shown using Co-IP assays in this research as well [27]. Furthermore, an FPIV aa sequence motif occurs as a late domain in the N-terminus of the NDV M protein. Mutation of the phenylalanine or proline within this FPIV motif to alanine results in reduced virus budding and decreased virus replication [28].

5. HN and F Protein

The NDV envelope contains two transmembrane glycoproteins, HN and F. The NDV HN protein is multifunctional, affecting processes of viral adsorption, internalization, replication, and dissemination [29]. The HN protein attaches the virus to sialic acid-containing receptors on the surface of a host cell. HN protein neuraminidase activity (NA) also mediates the cleavage of sialic acid from sugar side chains. The F protein mediates the fusion of the virion envelope with the cellular plasma membrane [30]. Both the HN and F proteins are essential for viral virulence and pathogenicity. As protective antigens, both HN and F proteins can induce the production of virus-neutralizing antibody response [31]. The F protein can protect birds from virulent NDV challenge. The HN protein has also been shown to induce protective immunity against NDV infection in chickens, although the neutralizing antibodies show lower titers. Additionally, monoclonal antibodies against F protein neutralize NDV more effectively than monoclonal antibodies against HN protein [32].

The F protein is a class I transmembrane protein, and the precursor exists in the form of F_0 [33]. The precursor protein F_0 is cleaved by cellular proteases into two subunits, F_1 and F_2. This F_0 cleavage is necessary for virus infection. The transmembrane (TM) domains of viral F proteins play a vital role in NDV F protein fusion activity [34]. The oncolytic activity of NDV has been traced to the minor multifunctional protein HN, and the HN protein has been shown to induce apoptosis in chicken embryo fibroblast cells [35].

The head and stalk region of the NDV HN protein functions in promoting membrane fusion. In this region, tetramer tails have an up process, which is called the hinge structure [36]. The role of the highly conserved amino acids in the head region of the HN protein is critical, and changing various amino acids in this region disables the F-promoting ability of the HN protein and inhibits syncytia formation. An analysis of HN globular head structure shows that the NA activity site is associated with a β-sheet propeller motif [37]. The binding of the HN stalk region to sialic acid promotes an F protein conformational change and activates the fusion of the viral envelope with

cell membranes [38].

Avirulent NDV strains, including Queensland V4, Ulster, and D26, possess HN proteins 616 aa long with an additional 39 or 45 aa at the C-terminus. These HN proteins appear to be precursors that can become bioactive after the removal of additional residues at the C-terminus [39]. In contrast, most NDV virulent strains have HN protein lengths of 577 or 571 aa. Hence, no residue removal is required for these HN proteins for full biological function. Notably, the shortest HN protein length observed is 571 aa, and this has only been found in velogenic and mesogenic strains. Therefore, the length of the NDV HN protein is assumed to closely relate to the virulence of the virus [40].

An NDV lentogenic strain, LaSota-based recombinant virus was generated with the 45 aa extension in the HN protein C-terminus, and this C-terminus extension was shown to not determine NDV enteric tropism [41]. However, recombinant virus virulence was decreased in this study compared with that of its parental virus, suggesting that the additional HN protein C-terminus 45 aa may be involved in virulence regulation. In another study of C-terminal extension effects, a recombinant virus with a C-terminus 45 aa insert derived from the lentogenic PHY-LMV42 strain was introduced into the mesogenic NDV Anhinga strain and rescued by reverse genetics techniques. The virulence of the recombinant virus and its parental strain was determined by mean death time (MDT) and an intracerebral pathogenicity index (ICPI). Their results indicated that the HN protein C-terminal extension may reduce virulence in virulence deficient NDV strains [42]. We also studied effects associated with NDV HN protein length diversity and biological properties. Our results show that NDV tissue tropism, replication ability, and pathogenicity are all closely associated with the origin of the HN protein [43].

6. L Protein

The L protein is 2,204 aa long with a molecular weight of 250 kDa. It is the largest NDV protein, containing almost all catalytic activities related to viral replication, transcription, and translation. The L gene may enhance virulence by improving viral gene replication levels. Recombinant L gene replacement chimeric viruses were compared with the parental virus, and significant changes in biological characteristics reveal that the L protein has a particular effect on virulence [44]. We previously demonstrated that NDV's envelope-associated proteins contribute significantly to genotype VII NDV virulence, and its polymerase-associated proteins also have a significant effect on viral pathogenicity. In the same research we showed that the L protein is a significant contributor to NDV virulence among the polymerase-associated proteins when combined with homologous NP and P proteins [45]. Notably, NP encapsulates the entire genomic RNA and the L protein works as an RdRp, combining with the P protein to form a complex that recognizes RNP for transcription and replication [46]. However, those specific domains of the L protein that determine virulence remain to be investigated.

The L protein, as a multifunctional protein in the process of virus proliferation, plays several vital roles. Due to its conservation, the L protein has become the first target protein to be investigated for antiviral drugs in other paramyxoviruses.

7. Conclusions

More and more studies have illustrated that NDV virulence is determined by the single and/or multiple roles of both structural and non-structural proteins. For NDV, the multi-basic amino acid cleavage motif of the F protein is an indispensable prerequisite for virulence. The other five structural proteins are also critical to virulence. Furthermore, other factors such as 5′ untranslated regions (UTRs) or intergenic sequences (IGSs) are involved in determining the virulence of NDV [47]. However, we must note that different and various contributions from these virulence factors may be associated with particular strains. As seen with velogenic strains, the F protein cleavage site is much more important than all other factors including the replication complex and noncoding regions. Another point to consider is the validity of using the ICPI as a criterion for determining virulence [46]. In conclusion, this review discusses NDV viral protein functions and those proteins' effects on virulence. An enormous amount of knowledge about ND and its causative virus has been obtained and deduced recently, as befits such a significant disease. However, it remains as an influence on the worldwide poultry industry, either as a continuing disease problem or as a constant threat.

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