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Avian influenza virus NS1
A small protein with diverse and versatile functions

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Abbreviations: 2'-5' OAS, 2'-5' oligoadenylate synthetase-RNase; AIV, avian influenza virus; AP1, activator protein 1; CEF, chicken embryo fibroblasts; CSPF30, the cleavage and polyadenylation specificity factor 30; DIVA, differentiating between infected and vaccinated animals; ED, effector domain; eIF4GI, eukaryotic translation initiation factor 4GI; HA, hemagglutinin; HP, highly pathogenic; IFN, interferon; IRF-3, interferon regulatory factor-3; M, matrix; MDCK, madin darby canine kidney; NA, neuraminidase; NFκB, nuclear factor kappa B; NLS, nuclear localization signal; NoLS, nucleolus localization signal; NP, nucleoprotein; NS, non-structure; P13K, Phosphatidylinositol 3-kinase; PA, polymerase acidic; PABP, poly(A)-binding protein; PB, polymerase basic; PDZ, postsynaptic density protein 95, drosophila disc large tumor suppressor, and zonula occludens 1 protein; PKR, protein kinase R; RBD, RNA binding domain; RIG-I, retinoic-acid inducible gene I; TNF-α, tumor necrosis factor alpha

Avian influenza viruses (AIV) of H5N1 and H9N2 subtypes have zoonotic and pandemic potential. 377 out of 633 human infections with H5N1 virus were fatal and human infections by H9N2 virus were infrequently reported to the World Health Organization.[1,2] Some H9N2 viruses either possessed genes similar to the H5N1 virus[3,4] or were claimed to donate gene segments to H5N1 virus.[5] Both features were reported in the Pakistani H9N2 viruses that have been recorded to be genetically similar to H5N1 isolated in Hong Kong in 1997[6] and also pertained a gene segment encoding the non-structural proteins (NS) almost identical to the co-circulating H5N1.[7,8] Therefore, it is important to compare biologically the impact of NS1 protein on replication and within-host cell dynamics of H5N1 and H9N2 viruses which have been recently studied by Munir and coworkers[8] and published in this issue of *Virulence*.

NS1: Is it More Vulnerable to Reassert than Other AIV Gene Segments?

The genome of AIV contains eight gene segments; polymerase basic 2 (PB2, segment 1), hemagglutinin (HA, segment 4), nucleoprotein (NP, segment 5) and neuraminidase (NA, segment 6) encode only one protein, whereas the PB1 (segment 2), polymerase acidic (PA, segment 3), the matrix (M, segment 7), and NS (segment 8) encode two proteins PB1 and PB1-F2, PA and PA-X, M1 and M2, and NS1 and NS2, respectively. Co-infection of the host cell with two or more AIV subtypes results in an exchange of gene segments designated as "reassortment" resulting in the emergence of novel viruses which may differ from their parent viruses in their ability to replicate and transmit between animals and humans.[9] Reassortment is a well-known genetic trait of influenza viruses resulting in new viruses causing pandemics in 1918, 1957, 1968, and 2009. In these events, the pandemic viruses contained genes from influenza viruses of swine and/or avian origin.[10]

There are cumulative data on the reassortment of NS gene segments between different AIV, particularly the H5N1 subtype. In their recent study, Munir et al.[8] raised this question again by reporting an H9N2 from backyard birds in Pakistan that carries the NS segment of H5N1 virus. Reassortment of the NS gene segment was observed within different clades of H5N1 in Hong Kong in 2000,[11] Thailand in 2004–2008,[12] Nigeria in 2006–2007,[13] and Vietnam in 2010–2012.[14] Intriguingly, three AIV subtypes, namely H1N1, H5N1, and H5N3, isolated from wild mallards in Belgium in 2008 had identical NS gene segments.[15] Similarly, A/turkey/Ontario/7732/1966 (H5N9) acquired its NS gene from a contemporary H5N1 virus,[16] and H6N2 and H6N8 from ostrich in South Africa acquired their NS genes from an H9N2 virus which was also closely related to contemporary H5N1 viruses in Asia.[17,18] Although rare, reassortment of NS from different H9N2 clades was also reported in China in 2002.[19] Moreover, NS of avian origin was introduced into swine influenza H1N1, H1N2, and H3N2[20] and equine H7N3 after 1973 as well as H3N8.[21] Whether variations in the frequency of reassortment exist in influenza genome segments, particularly NS of the H5N1 virus merits in-depth investigation.

NS: The Smallest Influenza Gene Segment with Multiple, Sometimes Overlapping, Functional Domains

The NS segment is the smallest AIV gene segment encompassing about 890 nucleotides. The NS1 protein of AIV contains between 124 and 237 amino acids, but the vast majority specifies 230 amino acids.[22] In H5N1 viruses deletion within the NS1

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Table 1. Molecular anatomy of influenza virus NS1 protein

Position	Structure	References
1–73	RNA binding domain	27
74–230/237	Effector domain	27
35–38	Nuclear localization signal 1 (NLS1)	85
35, 38, and 41	Nuclear localization signal 1 (NLS1)	86
38 and 41	RNA binding motifs	87
38 and 41	RNA helicase binding sites of the retinoic acid-inducible gene I (RIG-I)	62
1–81	Poly(A) binding protein I (PABPI) binding domain	27
81–113	Eukaryotic translation initiation factor 4GI (eIF4GI) binding domain	30
89 and 93	p85b binding domain	66 and 88
103 and 106	Cleavage and polyadenylation-specific factor 30-kDa subunit (CPSF30) binding domain	36
159 and 162	p85b binding domain	64
186	Cleavage and polyadenylation-specific factor 30-kDa subunit (CPSF30) binding domain	27
191–195	Cleavage and polyadenylation-specific factor 30-kDa subunit (CPSF30) binding domain	37
73–237	Staufen protein binding domain	27
123–127	Protein kinase R (PKR) binding domain	52
138–147	Nuclear export signal (NES)	86
148, 152, and153	Nuclear export signal (NES)	31
164–167	A putative SH3 binding motif	64
207–212	Phosphatidylinositol 3-kinase (P13K) binding domain	65
212–215	A putative SH3 binding motif	64
218–232	Poly(A) binding protein II (PABPII) binding domain	62
219, 220, 231, and 232	Nuclear localization signal 2 (NLS2)	86
221	Nuclear localization signal 2 (NLS2)	31
221	Nucleolus localization signal (NoLS)	31
219, 220, 224, 229, 231, and 232	Nucleolus localization signal (NoLS)	86
227–230	Postsynaptic density protein 95, *Drosophila* disc large tumor suppressor, and zonula occludens 1 protein (PDZ) binding domain	45

protein was observed between aa positions 80–84 and frequently in the tail region, mostly residues 225 to 230.[23] On the other hand, NS1 of H9N2 contains almost 230 amino acids[24] with rare insertion or deletion. Two genetic alleles (groups) of NS1 of more than 30% diversity were described. Allele A represents viruses of avian and mammalian origins, and allele B mainly avian origin viruses.[21,22,25] Generally, allele A is more common than allele B.[25,26] Identity between the NS1 proteins of H9N2 and H5N1 subtypes was more than 88%.[24] In their study, Munir et al.[8] reported almost identical NS1 genes of H9N2 and H5N1 viruses each with a total length of 225 amino acids due to deletion of the ^{80}TMASV^{84} motif.

Structurally, the NS1 protein is composed of two functional domains, an RNA binding domain (RBD) and an effector domain (ED). The RBD is located within amino acids 1 to 73. It contains a nuclear localization signal (NLS1) and a poly(A)-binding protein site (PABPI). Thus, it binds to different RNA species (e.g., viral RNA, viral mRNAs, poly[A] RNA and double stranded RNA). The ED within amino acids 74 to 230 has specific regions to interact with several host factors and proteins (**Table 1**) including the cleavage and polyadenylation specificity factor 30 (CPSF30), eukaryotic translation initiation factor 4GI (eIF4GI), PABPII, p58b-subunit of phosphatidylinositol 3-kinase (P13K). In some viruses a second NLS and nucleolus localization signal (NoLS) exist.[27-30]

As a non-structural protein, NS1 is not present in virions but it is abundant in the nucleus of influenza virus-infected cells early during infection and also in the cytoplasm at later stages of the viral replication cycle.[30] In their publication, Munir et al.[8] showed that both NS1 proteins they studied localized primarily in the nucleus 24 h after transfection of human A549 cells. It is worth mentioning that both NS1 proteins studied by Munir et al.[8] had an amino acid difference in position 221 that was described as essential for the nuclear and nucleolus localization of the protein.[31]

NS1: Enhancement of Virus Replication

Enhancement of viral replication by NS1 protein is usually achieved by direct activation of mRNA translation through interactions with, for example, eIF4GI and PABPII.[32] Mutations or deletions in these domains significantly hampered

replication of influenza viruses, both in vitro and in vivo[33-35] due to an increased interferon (IFN) response and rapid elimination of the virus. Mutations at residues 103 and 106 of NS1 increased virus replication in tissue culture,[33,36] and deletion of amino acids 191 to 195 reduced the ability of the virus to antagonize IFN production in chicken embryo fibroblast cells.[37] Introduction of NS1 from an H5N1 into H7N1 altered host range and tissue tropism, increased suppression of the host immune response and influenced virus replication in cell culture.[35,38,39] In contrast, NS1 reassortant viruses of H5N1 subtypes did not result in alteration of replication, tropism or pathogenicity of the viruses in experimentally infected ducks.[40] Munir and coworkers[8] showed in their study that both NS1 proteins, due to their high genetic relatedness, did not differentially affect transfection of H5N1 or H9N2 in different cell cultures.

NS1: A Virulence Marker

Virulence of influenza viruses is a multigenic trait, where mutations in more than one gene may be required to modulate severity of the disease in a host. NS1, in addition to other genes, was identified as a virulence determinant of the Spanish pandemic H1N1 from 1918–1919.[41,42] H5N1 virus that had a D92E mutation or a deletion of residues 80–84 exhibited high virulence in chickens and mice.[23] Mutations at residues 103 and 106 probably destabilize the CPSF30 binding pocket of NS1, and in an H5N1 or H1N1 enhanced virulence and altered brain-lung tropism in mouse model.[33,36] Deletion of amino acids 191 to 195, corresponding to the CPSF30 binding domain, attenuated swine-origin H5N1 virus in chickens.[37] Introduction of NS1 from an H5N1 into H7N1 increased virulence in mice and chicken embryos.[35,38,39] Moreover, a sequence motif at the C-terminal end of NS1, "ESEV" or "EPEV" in AIV or "RSKV" or "RSEV", in human H5N1 influenza viruses, in addition to interaction with PDZ-domain (postsynaptic density protein 95, *Drosophila* disc large tumor suppressor, and zonula occludens 1 protein) involved in cellular signal transduction pathways, was considered a species-specific virulence marker.[43-45] The studies conducted by Munir and coworkers[7,8] indicated that the NS1 of the two Pakistani H5N1 and H9N2 viruses had infrequent "ESKV" C-terminal PDZ motif with no clear effect on the pathogenicity after intravenous injection of 6-week-old chickens. Recently, highly pathogenic (HP) AIV H5N1 encoding NS1-ESKV in conjunction with NS1-F138Y caused local infection in mice respiratory tract, with mutations in both sites increasing virulence and resulting in systemic infection.[46]

NS1: A Host Range Determinant

Although species-specificity of the NS1 gene segment was reported earlier, the NS gene may not play an important role for host range restriction.[21] There was no association between a gull-specific NS1 lineage and HA gene which may indicate compatibility of gull-specific NS with HA of different AIV. In contrast, host restricted genetic signatures were reported frequently from the NS1 genes of H13 and H16 AIV subtypes.[47] HPAIV H7N1 containing NS1 from HPAIV H5N1 replicated at lower level in tracheal organ culture of chickens and turkeys,[35] and changed the replication dynamic and the host cell responses in mammalian cells[38] assuming host-specific variations. Among other proteins, concurrent mutations in the NS1 of an H9N2 were observed during adaptation to mice.[48] Moreover, AIVs that harbor allele B replicate poorly in the respiratory epithelial cells of primates[25,26] and efficiently on duck cells; conversely, the A allele is advantageous for replication in cells from chickens and turkeys origin.[49] Human influenza viruses, except the pandemic H1N1 viruses in 1918–1919 and 2009, mostly encode NS1 proteins with T215 but AIV, including H5N1 viruses, encode P215.[50] The PDZ domain 227–230 motif as described above also represents a species-specific genetic marker.[43] In the current study, Munir and co-authors[8] found that both NS1s, belonging to the A allele, supported growth of the viruses on chicken embryo fibroblasts (CEF) as well as on A549 cells.

NS1: A Multifaceted Regulatory Protein

The NS1 protein interacts with viral and cellular proteins. Preferential interaction of NS with the viral ribonucleoprotein complex,[51] polymerase,[52] NP, and/or M proteins[53] is required for regulation of influenza virus replication. Also, associations of polymerase and NS1 mutations or NS1 and HA mutations play an essential role in pathogenicity of H5N1 in mammals.[54,55] Therefore, the compatibility of NS1 to support replication of two different viruses, H9N2 belonging to the G1-lineage and H5N1 belonging to Z-genotype clade 2.2, as reported by Munir and colleagues,[8] emphasizes the role of NS to generate diverse influenza viruses/variants with efficient replication in nature.

On the other hand, the main role of NS1 is to antagonize IFN which is accomplished through two pathways (reviewed in details in refs. 27 and 28). One mode is via binding different RNAs, particularly double stranded RNA, and subsequent inhibition of the pre-transcription pathway for activation of IFN due to inactivation of cellular sensors such as protein kinase R "PKR"[56], retinoic-acid inducible gene I "RIG-I"[57-59] and 2'-5' oligoadenylate synthetase-RNase "2'-5' OAS"[60]. The second mode is via interaction with a variety of IFN-induced cellular proteins/factors by specific, sometimes overlapping, regions (**Table 1**). Post-transcriptional inhibition of IFN production occurs via binding of the NS1 with CPSF30 and PABPII required for maturation and export of host mRNAs encoding antiviral proteins, including IFN mRNAs.[58] Moreover, NS1 protein blocks induction of IFN by inactivation of TNF-α induced nuclear factor kappa B (NFκB), dsRNA-induced activator protein 1 (AP1), and the transcription factor IRF-3,[41,61,62] and regulates the IFN-inducible genes (e.g., *Myxovirus*-resistance protein, interleukin-6).[27,28] Also, NS1 protein enhances the translation of viral mRNA through interaction with eIF4GI and PABPI.[63] It also binds a number of PDZ proteins.[45] Furthermore, activation of the PI3K/Akt-pathway, including binding to SH3 and/or p85b, by NS1 protein

to control apoptosis of the cells has been described.[46,64-66] In late stages of the viral replication-cycle, the NS1 protein may be indirectly involved in morphogenesis of the virus particles through binding the Staufen protein.[67,68] In their study, Munir et al.[8] investigated the effect of NS1 protein of both H5N1 and H9N2 viruses on the IFN-β production in human and avian cells. They found that both NS1 proteins inhibited the IFN production equally by inhibition of the transcription factors IRF3, AP-1, and NFκB, and downregulation of IFN transcription both at the pre-mRNA and mature-mRNA levels.

NS1: A Target for Vaccine and Antiviral Therapy

Several experimental studies have shown that attenuated vaccines based on alterations in NS1 are effective to control influenza A and B viruses in different hosts. Viruses expressing truncated, site-specifically altered or temperature-sensitive NS1, have been assessed as vaccines. These vaccines have been validated to prevent clinical disease and limit virus excretion in pigs,[69] horses,[70] mice,[71-74] ferrets,[34] macaques,[75,76] and turkeys.[77] In chickens, the truncation or absence of NS1 in inactivated vaccines not only afforded protection against viral infection, but also provided a tool to differentiate between infected and vaccinated birds (DIVA[73,78,79,80]) by detection of anti-NS1 antibodies. Nonetheless, most of these studies did not address the stability of the vaccine in a live vaccine format. Brahmakshatriya et al.[81] showed that NS1 truncated attenuated live vaccine in chickens is not stable and can revert to a wild-type phenotype after only five passages. Also, NS1 becomes an attractive target for development of new influenza antiviral drugs,[82] particularly the CPSF30 binding domain.[83] Some herbal oils inhibited NS1 protein function and subsequently decreased H1N1 replication in Madin Darby canine kidney (MDCK) cells.[84] On the other hand, cellular pathways, particularly those associated with NS1, assessed in similar studies to that of Munir et al.[8] may be targeted as alternative strategy for developing universal antiviral drugs/vaccines against influenza virus.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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