The non-structural (NS) gene segment of H9N2 influenza virus isolated from backyard poultry in Pakistan reveals strong genetic and functional similarities to the NS gene of highly pathogenic H5N1

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Apart from natural reassortment, co-circulation of different avian influenza virus strains in poultry populations can lead to generation of novel variants and reassortant viruses. In this report, we studied the genetics and functions of a reassorted non-structural gene (NS) of H9N2 influenza virus collected from back yard poultry (BYP) flock. Phylogenetic reconstruction based on hemagglutinin and neuraminidase genes indicates that an isolate from BYP belongs to H9N2. However, the NS gene-segment of this isolate cluster into genotype Z, clade 2.2 of the highly pathogenic H5N1. The NS gene plays essential roles in the host-adaptation, cell-tropism, and virulence of influenza viruses. However, such interpretations have not been investigated in naturally recombinant H9N2 viruses. Therefore, we compared the NS1 protein of H9N2 (H9N2/NS1) and highly pathogenic H5N1 (H5N1/NS1) in parallel for their abilities to regulate different signaling pathways, and investigated the molecular mechanisms of IFN-β production in human, avian, and mink lung cells. We found that H9N2/NS1 and H5N1/NS1 are comparably similar in inhibiting TNF-α induced nuclear factor κB and double stranded RNA induced activator protein 1 and interferon regulatory factor 3 transcription factors. Thus, the production of IFN-β was inhibited equally by both NS1s as demonstrated by IFN stimulatory response element and IFN-β promoter activation. Moreover, both NS1s predominantly localized in the nucleus when transfected to human A549 cells. This study therefore suggests the possible increased virulence of natural reassortant viruses for their efficient invasion of host immune responses, and proposes that these should not be overlooked for their epizootic and zoonotic potential.

Introduction

The avian influenza viruses belonging to H9N2 subtype emerged for the first time in poultry (turkeys) in 1966. Since then, H9N2 viruses have been isolated from multiple avian species, particularly quails and land-based poultry throughout Asia, the Middle East, Europe and Africa. Their persistence and continuous circulation in avian species have led to the establishment of several lineages for example Ch/Bei/1/94 or Dk/HK/Y280/97, Y439, or Korean-like and Qa/HK/G1/97. The isolates from the former lineage have been isolated mainly from chickens and the latter predominantly from quails. There is a wealth of reports demonstrating the ability of H9N2 viruses to jump between terrestrial poultry and mammals (swine and humans). The reports of confirmed detection and isolation of H9N2 viruses from pigs and humans draw substantial attention to H9N2 viruses as potentially of zoonotic importance. It is further noted that H9N2 viruses that cause infection in humans show high similarity to influenza viruses of avian origin. In this scenario, it is believed that the presence of leucine-226 in the receptor-binding site of the hemagglutinin protein of H9N2, a typical residue for human H2 and H3 viruses, increases the human receptor specificity.

The H9N2 viruses circulating in Pakistan are phylogenetically similar to those of the G1 lineage of viruses (Qa/HK/G1/97) reported from Hong Kong. During 1999, the highly pathogenic avian influenza viruses (HPAIV) subtype H7N3 and H5N1 caused sporadic outbreaks in poultry in Pakistan. To reduce the impact of these viruses, an extensive vaccination program was implemented in Pakistan against H5, H7, and H9. These viruses have further crossed the species barrier and caused infection in...
Virulence

The NS segment is the shortest of the eight RNA segments of influenza A viruses which encodes for two proteins: NS1 and nuclear export protein (NEP). The NEP protein is primarily responsible for the nuclear export of RNPs. NS1 protein is structurally divided into two domains: RNA binding domain (RBD) and effector domain (ED). Both these domains of NS1 play essential roles in regulation of host cell functions, of which inhibition of interferon (IFNs) is well characterized. Anti-IFN actions of NS1 are primarily regulated by the inhibition of interferon regulatory factor 3 (IRF3), IRF7, nuclear factor κB (NFκB), and activating protein-1 (AP-1) transcription factors activation. Apart from these factors, direct interaction of NS1 with the tripartite motif containing protein 25 (TRIM25) leads to inhibition of retinoic acid inducible gene-1 (RIG-I) sensing, which results in the blocking of downstream signaling terminating at IFN-β production. Moreover, the NS1 suppresses the production of IFNs by binding to and sequestration of dsRNA, thus inhibiting the activation of the 2’-5’ oligoadenylate synthetase/RNase L system. In addition, NS1 has been shown to block protein kinase R (PKR), which is another antiviral protein induced by IFNs and tumor necrosis factor α. It has been characterized that the ED of NS1 protein binds to the cleavage and polyadenylation specificity factor (CPSF30) and poly(A)-binding protein (PABP). NEP, and thus inhibits the posttranscriptional modifications of pre-mRNA, which results in nuclear retention of cellular mRNAs, including the IFN-β mRNA. The ED of NS1 also interacts with a trimeric complex constituted of eukaryotic translation initiation factor elf4G1, PAB1, and elf4F, which is required for the translation of viral mRNA. Thus, NS1 protein employs a plethora of actions to cripple the host innate immune response and to facilitate viral replication.

The generation of reassortant viruses, by the exchange of genome segments between H9N2 and HPAI H5N1, leads to viral RNA recombinations that may change the pathogenicity, replication and transmission. Therefore, we attempted to screen NS1 proteins of H9N2 (H9N2/NS1) and HPAIV H5N1 (H5N1/NS1), in parallel, for their ability to regulate different signaling pathways, and investigated the molecular mechanisms in IFN-β production in human and avian cells.

Results

Phylogenetic analysis. In order to genetically characterize H9N2 virus isolated from backyard poultry flocks in northern Pakistan, all 8 gene segments were sequenced. The isolates were phylogenetically analyzed based on the hemagglutinin (HA), neuraminidase (NA), and non-structural (NS) gene segments, and compared with the representative strains of avian origin H9N2 viruses from Eurasian lineages. Earlier reports demonstrated that H9N2 viruses causing infection in poultry are mainly divided into three distinct lineages. These include Ch/BJ/1/94, Qa/HK/G1/97, and Y439 or Korean-like, which are represented by A/chicken/Beijing/1/94, A/Quail/Hong Kong/G1/97, and A/duck/Hong Kong/Y439/97, respectively. In the present study the phylogenetic tree based on the HA gene revealed that the isolate A/chicken/Pakistan/BYP/2010 clustered with the G1 lineage and made a distinct group together with recent isolates from the neighboring country Iran (Fig. 1A). Additionally, viruses isolated from China and the Middle East also clustered in the same group (G1) as that of A/chicken/Pakistan/BYP/2010. This isolate shown the highest nucleotide similarity with a previously characterized isolate from Pakistan. A corresponding tree topology was observed based on the NA gene tree, in which A/chicken/Pakistan/BYP/2010 clustered with previously reported Pakistani isolates and the isolates from the Middle East and China (Fig. 1B). In contrast, the phylogenetic analysis based on the NS gene segment revealed that A/chicken/Pakistan/BYP/2010 clustered with previously reported Pakistani isolates and the isolates from the Middle East and China (Fig. 1C). Interestingly, the NS gene segment of previously characterized Pakistani highly pathogenic H5N1 shows high nucleotide identity to the NS segment of H9N2.

Molecular characterization. It has been demonstrated that amino acid sequences connecting HA1/HA2 at the receptor binding site and glycosylation sites adjacent to receptor binding site not only play a central role in the switching viruses from low pathogenic to high pathogenic but also facilitate interspecies transmission. Comparison of the amino acid sequence of A/chicken/Pakistan/BYP/2010 to that of other Asian reference strains indicated that all of these have the same R-S-R-S amino acid motif, which is a typical characteristic of viruses with low pathogenicity. This motif has also been noted in the H9N2 viruses adapted to chickens in recent years in Asia and the Middle East. It is noteworthy that A/chicken/Pakistan/BYP/2010 has a lysine to arginine substitution at the –4 position of the cleavage site, and this substitution has been reported occasionally in H9N2 viruses. However, its biological importance is not reported yet. Eight potential glycosylation sites were predicted using the NetNGlyc 1.0 Server: NSTE, NPSC, NGTC, NVTY, NSTT, NISK, NGTY, and NES/ESC. The absence of two glycosylation sites at 206 and 218 has also been observed in isolates from the Middle East and in representative isolates of the G1 lineage, respectively. These alterations are associated with switching of influenza viruses to poultry. It is tempting to suggest that lack of these glycosylation may facilitate the selective adaptation of H9N2 viruses in poultry.

Three functional motifs have been described in the NA proteins that play essential roles in the pathogenicity of influenza viruses: the stalk length, sialic acid binding site, and glycosylation sites. The original two amino acid (at position 38–39 aa) and three amino acid deletions (at position 63–65 aa) in the NA stalk region as present in G1-like viruses (A/Quail/Hong Kong/G1/97) and Y280-like viruses (A/duck/Hong Kong/Y280/97)
were absent in A/chicken/Pakistan/BYP/2010. Further, the substitution associated with oseltamivir and zanamivir resistance was also not detected. In the sialic acid binding site, amino acids from 366 to 373 (IKKDSRTG), 400N, 403W, and 432K are conserved in G1-like and Y280-like viruses and in H9N2 viruses isolated from wild birds and adapted to poultry. All of these substitutions were found to be present in A/chicken/Pakistan/BYP/2010. It has been observed that glycosylation sites play a significant role in increased virulence of influenza viruses, either due to alteration in sialic acid activity or change in antigenicity. Comparison of glycosylation sites between G1-like H9N2 viruses and A/chicken/Pakistan/BYP/2010 demonstrated the presence of eight conserved potential glycosylation sites at: 44NSSK48, 61NITK65, 69NGTI73, 86NWSK90, 146NGTT150, 200NATA204, 234NGTC238, and 402NRSG406. Among them, the 44NSSK48 glycosylation site has also been reported in A/chicken/Hong Kong/G9/97 and A/chicken/India/2048/03.

Being a member of clade 2.2 of genotype Z of HPAI viruses H5N1 subtype, the NS gene segment of A/chicken/Pakistan/BYP/2010 showed highest nucleotide and amino acid identities to that of H5N1 viruses isolated from Pakistan, Afghanistan, Iran, India, and Russia during 2006 to 2007. The NS1 protein, a multifunctional protein that plays a fundamental role in virulence and pathogenicity of influenza viruses, showed a five amino acid deletion (80TMASV84 compared with PR8/34-CY040174), restricting its length to 225 amino acid. Additionally, a PDZ ligand at the C-terminus of NS1 proteins was found to be “ESKV”, typical of H5N1 viruses belonging to the Z genotype. All of the previously characterized residues were identical to NS1 protein of H9N2 (H9N2/NS1) and of HPAIV H5N1 (H5N1/NS1). However, an E221K substitution was observed, which is recently described as essential for the nuclear and nucleolus localization of NS1 protein of A/PR/8/34.43

Inhibition of dsRNA induced signaling pathways for type I IFN by H9N2/NS1 and H5N1/NS1. It has been described that reassortment of a single gene segment donates distinct phenotypic characteristics to novel variant viruses. However, the biological role of such reassortant genes has not been resolved in H9N2 subtypes. To this end, we initially investigated the ability of NS1 proteins of H9N2 and H5N1 to inhibit the induction of
These TFs include IRF3, nuclear factor κB (NFκB), and activator protein 1 (AP-1), which can be activated by intercellular dsRNA such as poly I:C. Taking the essential role of these TFs into consideration, we used a reporter plasmid that carries the luciferase gene under the control of the synthetic AP-1 binding site (pAP-1-Luc) and evaluated the effect of NS1 expression on dsRNA induced AP-1 activation. The results showed that NS1 proteins from both H9N2 and H5N1 significantly (P < 0.05) inhibited AP-1 activation when compared with the mock treated control (pAP-1 cont) (Fig. 2B).

Finally, we examined whether TNF-α mediated activation of the NFκB promoter is inhibited by expression of the viral type I IFN, a function that is well characterized for NS1 protein of influenza A viruses. For these purposes, expression vectors for NS1 were constructed encoding an N-terminal flag-tag for homogeneous detection of expression. The A549 cells were co-transfected with NS1 expression vectors and 4×IRF3 reporter plasmid, and stimulated with dsRNA (poly I:C) per ml (4×IRF3 and pAP-1) or 25 ng/ml of TNF-α (NFκB) or left untreated (cells). After an additional 24 h of incubation period, the cell extracts were prepared (A–C). CEF cells were transfected with corresponding constructs, and all the conditions were maintained as practiced for A549 cells. After 24 h of stimulation of (D) 4×IRF3 and (E) pAP-1 promoter with (F) 10 μg of dsRNA per ml or NFκB promoter with 25 ng/ml of TNF-α or untreated cells, the luciferase extracts were prepared. In both cell lines, the luciferase activity was measured using the Dual-Luciferase Assay System (Promega). The values in the mock-transfected cells were normalized and were set to 100% (4×IRF3 cont., pAP-1 cont., and NFκB cont.). Error bars indicate standard deviations. The data shown here are representative for 3 experiments with transfections performed in duplicate. *indicates a significant difference as determined by the Student t test, with P values of <0.05.

Figure 2. Inhibition of dsRNA or TNF-α induced pathways by overexpression of the NS1 protein of influenza A viruses. A 500 ng of expression plasmids for NS1 protein derived from H9N2 (H9N2/NS1) and H5N1 (H5N1/NS1) were cotransfected with 10 ng of pGL4.74 together with 500 ng of (A) 4×IRF3-Luc, (B) pAP-1-Luc, and (C) NFκB-Luc reporter plasmids or normalized with empty flag vector (cont.) or left untreated (cells). After 24 h post-transfection, the A549 cells were either stimulated with 10 μg of dsRNA (poly I:C) per ml (4×IRF3 and pAP-1) or 25 ng/ml of TNF-α (NFκB) or left untreated (cells). After an additional 24 h of incubation period, the cell extracts were prepared (A–C). CEF cell were transfected with corresponding constructs, and all the conditions were maintained as practiced for A549 cells. After 24 h of stimulation of (D) 4×IRF3 and (E) pAP-1 promoter with (F) 10 μg of dsRNA per ml or NFκB promoter with 25 ng/ml of TNF-α or untreated cells, the luciferase extracts were prepared. In both cell lines, the luciferase activity was measured using the Dual-Luciferase Assay System (Promega). The values in the mock-transfected cells were normalized and were set to 100% (4×IRF3 cont., pAP-1 cont., and NFκB cont.). Error bars indicate standard deviations. The data shown here are representative for 3 experiments with transfections performed in duplicate. *indicates a significant difference as determined by the Student t test, with P values of <0.05.
NS1 proteins of both H9N2 and H5N1 origin. It has been reported that TNF-α activates the signaling pathways culminating in the strong activation of NFκB.45 We evaluated the level of activation of NFκB in the presence of H9N2/NS1 and H5N1/NS1. As shown in Figure 2C, TNF-α strongly induced the NFκB promoter activation (pNFκB cont) and was significantly (P < 0.05) inhibited by the overexpression of both NS1 proteins. Therefore, we concluded that NS1 protein of H9N2 viruses has strong abilities to downregulate IFN-β production from all major possible ways when compared with HPAIV H5N1 subtype.

It is possible that the inhibitory role of NS1 protein might be species-specific; and to test the ability of NS1 to inhibit type 1 IFN in its homologs source, we examined these pathways in chicken embryo fibroblast (CEF). Under the same experimental conditions as that of A549 cells, both NS1 proteins of H9N2 and H5N1 were considerably better at blocking 4×IRF3, AP-1, and NFκB promoters in CEF as shown in Figure 2D–F, respectively. The H9N2/NS1 showed an inhibition response of 90%, 65%, and 80% whereas H5N1/NS1 showed an inhibition response of 83%, 70%, and 74% for 4×IRF3, AP-1, and NFκB promoter activities, respectively.

Expression and cellular localization pattern of NS1 proteins in A549 cells. To investigate whether both NS1s are homogeneously expressed, A549 cells were transfected with equal amounts of NS1 constructs or left untreated in a 6-well plate and lysed 24 h afterward. The results of western blotting (WB) using anti-flag antibodies indicated that both NS1s from H9N2 and H5N1 were expressed in high quantity and the expression level was comparable (Fig. 3A). To further examine the nuclear and cytoplasmic pattern, a highly sensitive technique named in situ PLA was applied. Under the same experimental conditions as WB, cells were paraformaldehyde fixed and stained with anti-flag and anti-β-actin antibodies. Analysis by in situ PLA indicated that NS1 proteins show high level of homology in terms of type I IFN inhibition and cytoplasmic expression pattern.

NS1 protein of H9N2 and H5N1 are equally potent in blocking IFN-β transcription. The previous results shown that NS1 is implicated in the inhibition of all transcription factors such as IRF3, NFκB, and AP-1. The end result of these TFs is the initiation of transcription of type I IFNs by binding to their respective PRDs. Next, we investigated whether the NS1 proteins of both subtype of influenza A virus carry the ability to block IFN-β transcription. Moreover, NS1 protein is involved in the inhibition of 3′-end processing of mRNA.27 To this end, we employed a quantitative real-time PCR (qPCR) to measure the level of both pre-mRNA and mature-mRNA for IFN-β.46 A549 cells were TRIzole lysed at specified time points of post-stimulation in the presence of expressed NS1 proteins or mock treated. The results of qPCR indicated that the NS1 protein of H9N2 significantly (P < 0.05) blocked the transcription of IFN-β when compared with mock treated-dsRNA stimulated (Poly I:C stimulated) control, and this property was also shared by the NS1 protein of H5N1 (Fig. 4A).

After the transcription of pre-mRNA, it undergoes post-transcriptional modifications, which are mainly mediated via cleavage and polyadenylation specificity factor 30 (CPSF30). NS1 protein has been demonstrated to directly interact with CPSF30 and facilitate the nuclear retention of cellular mRNAs, including the IFN-β mRNA.27 To investigate the implication of NS1 proteins in the processing of IFN-β mRNA, the level of mature-mRNA for IFN-β was quantified. As shown in Figure 4B, the results indicated that both NS1 proteins equally inhibited the processing of mature IFN-β mRNA. This inhibition was significantly (P < 0.05) lower compared with mock transfected but dsRNA-stimulated cells.

Contribution of NS1 proteins in an anti-viral state induced by IFNs. To investigate whether NS1 proteins could inhibit the type I IFN response, we used a VSV-GFP bioassay, which is based on the production of the robust IFN-mediated antiviral state that prevents the replication of VSV-GFP (Fig. 5A). For this bioassay, human A549 cells were used, as these cells have been widely

![Figure 3](image-url). Expression patterns of H9N2/NS1 and H5N1/NS1 proteins in subcellular compartments in A549. (A) The A549 cells were transfected with 500 ng of expression plasmids encoding NS1 from H9N2/NS1 and H5N1/NS1 or with empty flag vector (mock treated). After 24 h post-transfection, the cells were lysed and subjected to western blotting by stained with anti-flag and anti-β-actin antibodies. (B) Transfections were performed as demonstrated for western blotting. After 18 h of transfection, cells were fixed and processed for in situ PLA. The images reflect a predominant expression of NS1 proteins in the nucleus. Both H9N2/NS1 and H5N1/NS1 proteins showed comparable expression as seen in the western blotting. The images were taken at 20×.
studied to ascertain the virus-IFN interactions. A549 cells transfected with both NS1s of H9N2 and H5N1 supported similarly high levels of VSV-GFP multiplication (Fig. 5B and C). This suggested that the NS1 proteins of both viruses significantly inhibited type I IFN produced due to dsRNA stimulation. In contrast, mock-transfected A549 cells produced a strong anti-viral state, which prevented the multiplication of VSV-GFP, as predicted. Therefore, we concluded that NS1 proteins of H9N2 and H5N1 are involved in efficient inhibition of endogenous IFN-β synthesis and secretion. Furthermore, these results suggested that both NS1 proteins target the same mechanism(s) involved in IFN production.

Inhibition of dsRNA induced activation of IFN-β promoter. Following production, IFN-α/β initiates a positive feedback-production loop by binding to the IFN-α/β receptor (IFNAR) in both auto- and paracrine manners, leading to activation of IRF9, which binds to IFN stimulatory response element (ISRE) and establishes an anti-viral state. Taking this into consideration, we next monitored whether NS1 proteins interfered in the signaling of IFN. We employed a reporter assay based on ISRE promoter that is fused with firefly luciferase. For this purpose, A549 cells were transfected with ISRE reporter and pGL4.74 plasmids (renilla) as well as vectors encoding NS1 of H9N2, H5N1, or empty vector. Luciferase activities for both firefly and renilla were measured 24 h post dsRNA stimulation. The results suggested that both NS1s significantly (P < 0.05) inhibited the ISRE promoter activation to the level of 70% and 72% by H9N2/NS1 and H5N1/NS1, respectively (Fig. 6A). The empty vector did not show any effect on ISRE promoter inhibition. To estimate the action of NS1 protein on the

Figure 4. Inhibition of pre- and mature-IFN-β mRNA transcription in the presence of H9N2/NS1 and H5N1/NS1 proteins. A549 cells were transfected with H9N2/NS1 and H5N1/NS1 expression plasmids or left untreated (poly I:C stimulated and cells). After 24 h of post-transfection incubation period, the cells were either left unstimulated (cells) or stimulated with 10 μg of dsRNA per ml. Cells were TRIzole lysed and RNA was extracted at 0, 2, 4, 8, 16, and 24 h post-stimulation. Equal amounts of RNA from all time points were used to synthesis cDNA for (A) pre-mRNA (with 20 mer oligonucleotide) and for (B) mature-mRNA (with poly dT[20] primer). Both cDNAs were subjected to real-time PCR for IFN-β and β-actin mRNA quantification. The results were presented as fold increase in IFN-β mRNA using the 2-ΔΔT method. *Indicates significant differences at P < 0.05 (the Student t test) in 3 independent experiments.

Figure 5. The H9N2/NS1 and H5N1/NS1 proteins inhibit the IFN-β gene expression in VSV-GFP bioassay. (A) The stimulation of IFN-β by dsRNA is inhibited in the presence of NS1 proteins and thus leads to efficient replication of VSV-GFP virus. (B) A549 cells were transfected with 300 ng of expression plasmids encoding H9N2/NS1 and H5N1/NS1 proteins or left untreated (mock treated). After 18 h post-transfection incubation period, the cells were stimulated with 5 μg of dsRNA per ml. After an additional 24 h, the cells were infected with VSV-GFP (MOI of 2). Fluorescence was measured after 18 h of infection. The images were taken at 20×. The quantitative analysis of the infected cells effectively expressing replication of VSV-GFP in the presence of NS1 protein from both H5N1 and H9N2. Bars represent a non-significant difference at P > 0.05 (the Student t test) in 3 independent experiments.
Using the same experimental layout as described for A549 cells, is species specific, we further investigated this pathway in CEF. NS1) (both NS1 proteins (64% for H9N2/NS1 and 66% for H5N1/NS1) block nuclear translocation of IFN-β promoter, p125-luc reporter plasmid was used. The level of IFN-β promoter inhibition was also significant (P < 0.05) in both NS1 proteins (64% for H9N2/NS1 and 66% for H5N1/NS1) (Fig. 6B).

To exclude the possibility that this function of NS1 protein is species specific, we further investigated this pathway in CEF. Using the same experimental layout as described for A549 cells, we were able to draw the conclusion that both NS1 proteins of H9N2 and H5N1 were comparatively stronger in blocking ISRE, p125-Luc promoters in CEF (Fig. 6C). Correspondingly, the level of p125 promoter inhibition in CEF was also stronger than observed in A549 cells (Fig. 6D). The H9N2/NS1 showed an inhibition response of 90% and 80% on ISRE and p125 promoter activities whereas H5N1/NS1 showed an inhibition response of 80% and 70% for ISRE and p125 promoter activities, respectively.

H9N2/NS1 and H5N1/NS1 block nuclear translocation of IRF3. From the results shown in Figure 2A and D it is tempting to conclude that both NS1 proteins of avian influenza A viruses block the induction of IFN-β mRNA downstream of the activation of IRF3, most likely in the nucleus. It has been demonstrated that dsRNA leads to phosphorylation of IRF3 at serine396.44 Finally, we monitored the phosphorylation of IRF3 using antibodies specific for serine396. To investigate whether the phosphorylation of IRF3 is inhibited by the expression of NS1 proteins, A549 and MDCK cells were transfected with NS1s of the H9N2 and H5N1 subtypes of influenza virus or left untreated, for 24 h. Cells were fixed at 24 h post-dsRNA stimulation and probed with activated IRF3 using anti-pIRF3 (ser396). As shown in Figure 7, expression of H9N2/NS1 protein in A549 cells led to inhibition of nuclear translocation of IRF3. Similar results were observed when A549 cells were transfected with H5N1/NS1 protein. As expected, the phosphorylation and subsequent nuclear localization of IRF3 was not affected in mock treated cells. These results suggested that influenza A virus NS1 proteins are able to inhibit internal IFN induction pathways through inhibition of IRF3 activation and nuclear translocation.

**Discussion**

Isolation and characterization of H9N2 subtype of influenza A viruses have been demonstrated often in a well-adapted host, the quail. The G1-like lineage of H9N2 viruses has been found to be stable in quails, where limited reassortment events have been recorded.29 On the other hand, the genetic reassortment among avian influenza A viruses including the G1-like H9N2 lineage has predominantly been observed in both wild and domestic birds.12,29

The great wealth of reports demonstrating the continuous circulation of H9N2 viruses in Asian poultry highlights the potential of these viruses to evolve rapidly, and attain increased virulence. A recent report of increased replication of poultry-origin H9N2 viruses in a mouse model provides further evidence and sensitivity of human-avian contact.49 This situation becomes even more complicated when such interpretation appears in healthy backyard poultry (BYP) flock. The apparent masking of the clinical picture of the disease in asymptomatic BYP may increase the chance of human exposure, and these observations have further been confirmed by isolation of H9N2 viruses from humans and animals, predominantly from Southeast Asia.3,6,7,12,50,51

Generation of novel and reassortant H9N2 strains with NS gene segments of HPAIV H5N1 subtype is a direct indication of the essential role of the NS1 protein in pathogenicity, host range determination and cell-tropism. The NS1 protein is responsible...
for a plethora of cell regulatory activities that are essential for establishing strong anti-influenza response.\textsuperscript{13} Among these multiple functions, we focused in this study on exploring and comparing the role of NS1 proteins from H5N1 and H9N2 viruses in abrogating IFN production and action, which is the best-characterized function of NS1 protein. To achieve this, the luciferase reporter gene was used to monitor the activities of different signaling pathways that are directly associated with IFN production. In this model system, dsRNA (poly I: C) or TNF-\(\alpha\) were used as a stimulant for different downstream targets.\textsuperscript{35} Apart from the NS1 protein, two other viral proteins have recently been demonstrated to be involved in inhibition of IFN-\(\beta\) production.\textsuperscript{32,33} Since this model is entirely based on the functional interference of the NS1 protein, the possibility of involvement of other IFN-inhibiting factors of influenza viruses such as PB2 and PB1-F2 can conveniently be excluded, and hence exclusively demonstrate the function of NS1 protein.

In this study, we monitored the role of the NS1 protein of H5N1 like-recombinant H9N2 viruses and by virtue of identity, IFN-suppressive properties were compared with HPAI subtype H5N1. Owing to the high similarity of both NS1 proteins, we were able to demonstrate that H9N2/NS1 and H5N1/NS1 are equally potent in mediating resistance to the cellular IFN system, which would likely favor the H9N2 viruses to replicate efficiently in the presence of a functional IFN system. The anti-IFN function of NS1 protein was found to be strong enough to leave no possibility for the cells to establish resistance against influenza viruses. A potent inhibition of all of the components of the enhansosome including IRF3, NF\(\kappa\)B, and AP-1, an essential factor of IFN-\(\beta\) mRNA transcription, strongly suggests the functional identity between NS1 proteins of both subtypes of influenza viruses (H9N2 and H5N1).\textsuperscript{14,15} However, it would be of interest to compare the IFN-abrogating abilities of NS1 protein of naive (non-recombinant) H9N2 in parallel to H5N1-like H9N2/NS1 and H5N1 NS1 proteins to estimate the level of advantage of the NS gene segment acquisition in recombinant H9N2 influenza A viruses.

It has been demonstrated that the NS1 protein of influenza A viruses inhibits IFN-\(\beta\) production at multiple steps.\textsuperscript{36} Therefore, we followed the whole pathway of IFN transcription, synthesis and signaling terminated at ISRE elements. The results demonstrated that both NS1s are able to downregulate IFN transcription both at the pre-mRNA and mature-mRNA levels, suggesting their involvement not only in mRNA processing, as reported before,\textsuperscript{16,27,56,57} but also in the transcription of IFN-\(\beta\) mRNA. We further analyzed their effect on IFN-\(\beta\) gene expression, a step that follows IFN-\(\beta\) transcription. As expected, the level of IFN-\(\beta\) protein was also suppressed in the presence of any of the NS1 proteins. The IFN-\(\beta\) protein is secreted from the cell and acts on IFN-\(\alpha/\beta\) receptors (IFNAR) in both an auto- and paracrine manners, leading to activation of IRF9, which binds to the IFN stimulatory response element (ISRE) and establishes an anti-viral state through expression of IFN-stimulated genes (ISGs).\textsuperscript{47} It is tempting to hypothesize that these NS1 proteins might differ in their abilities to interact and inhibit the Janus kinase/signal transducer and activator of transcription protein (JAK-STAT) pathway, since the NS1 has recently been demonstrated to upregulate the suppressor of cytokine signaling (SOCS) 1 and SOCS3, which are reported to be strong inhibitors of the JAK-STAT pathway.\textsuperscript{58} In addition to blocking IFN-\(\beta\) transcription and IFN-\(\beta\) gene expression, the NS1 protein of both H9N2 and H5N1 was found to block the IFN signaling, as demonstrated by the use of a reporter assay carrying promoter for ISRE.

Inhibition of nuclear localization of IRF3, as a strategy to abrogate IFN mRNA transcription by NS1, has also been evaluated.\textsuperscript{16,59} Consistent with previous reports, it was found that both NS1 variants inhibited the nuclear localization of IRF3 transcription factor. Based on this, we can conclude that IRF3 indeed contributes to the induction of IFN-\(\beta\), but it is considered insufficient for complete IFN-\(\beta\) transcription and requires NF\(\kappa\)B and ATF-2/c-jun (AP-1) transcription factors to cooperate in the enhanceosome.\textsuperscript{53,10,20,37} Consistent with this idea, the results also indicated that both NS1 proteins equally inhibit NF\(\kappa\)B and AP-1 promoters.

Currently, the correlation of expressed NS1 protein in either the cytoplasm or nucleus to that of its function has been demonstrated. It has been revealed that the expression pattern of NS1 protein is a species-dependent character,\textsuperscript{60} whereas another study demonstrated that the amino acid at position 221 in the C-terminus is potentially important for nuclear localization (NL) and nucleolus localization signals (NoLS).\textsuperscript{61} The sequence analysis of both NS1 proteins showed that the NS1 protein of H9N2 carries lysine (K) at position 221 whereas NS1 protein of H5N1 carries isolinuc (I) at this position (Fig. S1). Despite these substitutions, there was no difference in the expression and cellular localization pattern, in accordance with previous reports.\textsuperscript{61} Although no correlation has been reported for the NS1 protein localization and virus replication. The localization pattern was found to be different (cytoplasmic) during infection of influenza virus.
viruses than transfection of NS1 constructs (nuclear), which warrants further investigations. Moreover, it is required to interpret that H5N1 NS1 would provide a replication advantage for the H9N2 low pathogenic strains in the absence of gene exchange experiments in the virus context.

In conclusion, the apparent natural selection and persistence of viruses containing a mixture of influenza virus genes demonstrates the importance of optimal gene constellations that permit efficient replication and interspecies transmission. It is further conceivable that possession of the NS gene segment by low pathogenic H9N2 viruses increases the capacity of the viruses to adapt to new hosts and environments with increased pathogenicity. Furthermore, this fact may establish a process of evolution, which would primarily be shaped by the involvement of single gene, and ultimately facilitate the viruses to jump from birds to humans and other mammals, thus highlighting their epizootic and zoonotic potential. The genesis of new influenza virus strains principally through reassortment but also by host adaptation does present theoretical opportunities for the production of pandemic strains, so their potential role in inter-species transmission remains important. Apart from this activity (IFN-suppressive) of NS1 protein, its implication in virus replication, in accumulation of viral RNA and viral ribonucleoprotein export and consequent involvement in different stages of virus life cycle should not be overlooked.

Material and Methods

Virus collection, isolation, typing, and pathogenicity assessment. Samples were collected from backyard poultry flocks, and the virus was isolated in 10-d-old embryonated hens’ eggs at the Veterinary Research Institute (VRI), Lahore, Pakistan. Initial virus typing was performed using standard hemagglutination-inhibition and neuraminidase inhibition assays according to the manufacturer's instructions. Allantoic fluids from the samples showing high hemagglutinin titer were assessed for intravenous pathogenicity index (IVPI) in 6-week-old chickens to determine the pathogenicity, as previously described. For genome detection and characterization, the allantoic fluid was stored on QIAcard FTA Indicator Four Spots, which preserved the nucleic acids and inactivated the virus. The samples were shipped at ambient temperature from Pakistan to the Swedish University of Agricultural Sciences (SLU), for processing.

Nucleotide sequencing and phylogenetic analysis. The eluted RNA from QiaCard FTA Indicator (Qiagen) was used for viral gene amplification and sequencing, as described previously. All 8 gene-segments of this isolate were genetically characterized, phylogenetically analyzed, and compared with virus sequence data available in GenBank. The genetic pattern was determined and phylogenetic trees were constructed using the neighbor-joining method (Kimura 2 parameter) with 2000 bootstrap replicates using the Molecular Evolutionary Genetics Analysis (MEGA, version 5) software package (CEMI). To confirm the genetic pattern demonstrated by the neighbor-joining method, trees were constructed using Bayesian Inference with the program MrBayes version 3.1.2. Two independent Monte Carlo Markov (MCM) chains were executed and sampled every 1000 generations using the default parameters of the priors. Trees saved in this last step were used to construct a majority rule consensus tree. The NetNGlyc 1.0 Server was used to predict the potential glycosylation sites in hemagglutinin (HA) and neuraminidase (NA) proteins of H9N2 isolate.

Cell lines and antibodies. A549 cells, a type II alveolar epithelial cell line from human adenocarcinoma (ATCC, CCL-185), Madin Darby Canine Kidney (MDCK) (ATCC CCL-34) and Chicken Embryo Fibroblasts (CEF) (ATCC CRL-1590) were grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin per ml, and 100 mg streptomycin per ml. Monoclonal antibodies to β-actin were obtained from ProSci and pIRF3 ser396 from Millipore. The NS1 protein levels were detected using a monoclonal anti-FLAG antibody from Sigma-Aldrich. Donkey anti-mouse IgG secondary antibodies (Jackson Immuno Research, Europe Ltd) were kindly provided by Dr. Claudia Baule (National Veterinary Institute [SVA]). The TNF-α human recombinant protein, as NFkB stimulant, was purchased from Invitro.

Expression and reporter plasmids. The pCMV-FLAG mammalian expression vector (Sigma-Aldrich) possessing an N-terminal flag tag was used to construct NS1 clones from avian origin H9N2 (A/chicken/Pak/BYP/2010 [H9N2/NS1]) and from a Swedish isolate of highly pathogenic avian influenza virus H5N1 (A/tufted-duck/Sw/V789/06 [H5N1/NS1]). The open reading frames of both NS1s were amplified from full-length cDNA of segment eight using NS1-XbaI-Fw: 5’-ATATTCTAGA GGATTCCAAC ACTGTGTCAA GC-3’ and NS1-KpnI-Rev: 5’-TACTGTACC CGCAGTCTTT CATCAACCA TC-3’. The resultant products were cloned into pCMV-FLAG vector in frame, and confirmed by sequencing.

The firefly luciferase reporter constructs were used containing one of the following inducible promoters: pISRE-Luc, containing IFN-stimulated response element (ISRE) promoter; NFκB-Luc, containing an NFκB promoter; and AP-1-Luc, containing an AP-1 promoter (Clontech Laboratories, Inc.). The pGL4.74 plasmid (containing a renilla luciferase gene) was used as transfection control to standardize all the reporter assays (kindly provided by Magnus Johansson, School of Life Sciences, Södertörns University). The 4×IRF3-Luc plasmid contains 4 copies of the IRF-3-binding positive regulatory domain (PRD) I/III motif of the IFN-β promoter upstream of the luciferase reporter gene, and was kindly provided by Dr S Ludwig (Institute of Molecular Virology, Centre for Molecular Biology of Inflammation [ZMBE]). An IFN-β promoter-driven luciferase reporter gene (p125-Luc) was from Hongxia Li (Wuhan Institute of Virology Chinese Academy of Sciences).

Transfection and reporter assays. All of the transfections were performed using Lipofectamine 2000 (Invitrogen) in A549, MDCK, and CEF cells according to the manufacturer’s instructions. At 80% confluence the 24-well plates were co-transfected with 500 ng of each of the reporter plasmids (pISRE, NFκB, pAP-1, p125, 4×IRF3) and the expression vector (H9N2/NS1 and H5N1/NS1), using Lipofectamine 2000 with a ratio of 1:1. As a positive control, each of the reporter plasmids was co-transfected with an empty FLAG vector. Ten nanograms of pGL4.74

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plasmid was used as a transfection control. Twenty-four hours post-transfection, the cultures were stimulated with 10 μg of dsRNA (poly I:C) (Invivogen) per ml or 25 ng/ml of TNF-α. Twenty-four hours post-stimulation, the cells were lysed and the activities of firefly and renilla luciferases were determined by a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. The data represent firefly luciferase activity normalized to renilla luciferase activity. The positive signaling controls were set to 100% and level of inhibition was calculated as a percentage. All assays were performed in triplicate and the results expressed as means ± the SD.

In situ proximity ligation assay. In situ proximity ligation assays (PLA) was performed with the Duolink in situ PLA kit (Olink Biosciences), as described previously20 with some modifications. Briefly, A549 cells were seeded in 8-well chambers (VWR international) for 24 h, followed by transfection with 300 ng of NS1 constructs using Lipofectamine 2000 or left untreated. Cells expressing NS1 were fixed with 1% paraformaldehyde followed by permeabilization and blocking. After the blocking, the cells were stained with primary antibodies against flag tag. After removal of unbound antibodies, the cells were incubated with anti-rabbit PLUS and anti-rabbit MINUS proximity-probes at 37 °C for two hours. Thereafter, all of the steps were followed as described before.20 Slides were visualized using a standard immunofluorescence microscope (Nikon) with suitable filter for Texas Red (PLA signals) and Hoechst 33342 (nuclear) staining.

Western blotting. All of the transfections for western blot analysis were performed in 24-well plates. At the indicated times, cells were washed and lysed using lysis buffer. Concentration and quality of protein were measured using Nanodrop ND1000 (Nanodrop Tec.). A total of 50 μg of lysate was separated by sodium dodecyl sulfate-PAGE (SDS-PAGE) in Ready Gel J 7.5% (Bio-Rad) and then transferred to polyvinylidene difluoride (PVDF) membrane (GE Healthcare). The membranes were incubated in blocking buffer (PBS, 2% [wt/vol] bovine serum albumin) at room temperature for 1 h with slow agitation, followed by incubation in primary antibodies against flag tag and β-actin diluted in 2% BSA at 4 °C overnight. After intensive washing with PBST (PBS, 0.2% Tween 20) membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies for 2 h at room temperature with continuous agitation. The blots were developed by an ECL advance kit from GE Healthcare, and visualized by ChemDoc XRS system from Bio-Rad with Quantity One® software.

VSV-GFP IFN bioassay. A549 cells were transfected with 300 ng of the indicated expression plasmids using Lipofectamine 2000 (Invitrogen) or left untreated. Eighteen hours post transfection, cells were stimulated with 5 μg of dsRNA per ml for 24 h and then infected with vesicular stomatitis virus (VSV) expressing GFP (VSV-GFP) with an MOI of 2. The fluorescence intensities were measured at 18 h post infection. Images were taken using an immunofluorescence microscope (Nikon). The VSV-GFP was a kind gift of John Rose. Quantitation was performed by microscopic counting of the infected cells.

Quantitative measurement of pre- and mature-IFN-β mRNA. To measure the level of pre- and mature-IFN β mRNA inhibition by the NS1 protein of both H9N2 and H5N1, A549 cells were seeded into 6-well plates. Cells were transfected with the indicated NS1 constructs or left untreated. Twenty-four hours post-transfection, cells were either mock treated or stimulated with 10 μg of dsRNA per ml. The cells were lysed using TRizol Reagent (Invitrogen) at 0, 2, 4, 8, 16, and 24 h post-stimulation. RNA was extracted using RNeasy Mini kit and treated with DNase using a on column DNase Digestion kit (Qiagen) to remove any DNA contamination. To measure the IFN-β pre-mRNA, the total RNA was reverse transcribed into cDNAs using 20-mer oligonucleotide (5'-CACTAATAG GTACTTGGA-3') which target the sequence upstream to poly(A) tail.46 For mature-IFN-β mRNA quantification, the total RNA was reverse transcribed using Oligo(dT)20 (Invitrogen) which target the poly(A) tail of mature IFN-β mRNA. Both pre- and mature-mRNAs were quantified using Brilliant II SYBR Green QRTPCR Master Mix kit (Agilent Technologies) with oligonucleotides for IFN-β (Fw 5'-CAGTCTGAC CTGAAAAGAT ATTATG-3' and Rev 5'-CCTGGGACCAT AGTCAGAGTG GAAATC-3'), as per the manufacturer’s instructions. To normalize the loading cDNA, β-actin was run in parallel at all time points using forward (5'-TGGGTCAGAA GGACTCCTAT G-3') and reverse primers (5'-AGAAGAGGCTA TGAGCTGCCT G-3').

IRF-3 nuclear translocation assay. A549 and MDCK cells were seeded in 8-well chamber slides (VWR international) and transfected for 24 h with NS1 constructs of H9N2 and H5N1 using Lipofectamine 2000 (Invitrogen) at a ratio of 1:1 per well. At 24 h incubation post transfection, phosphorylation of IRF3 was stimulated with 10 μg of dsRNA. At 24 h post stimulation, cells were fixed in 1% paraformaldehyde for 40 min. Then the cells were stained with anti-pIRF3 (ser396) rabbit monoclonal antibodies, followed by incubation with Cy3-mouse anti-rabbit IgG as secondary antibodies. The nuclear localization of actin was performed using an immunofluorescence microscope (Nikon) using suitable filters for Texas red (IRF3) and DAPI (nuclear) staining.

Statistical analysis. A Student t test was employed to ascertain the statistical significance. P values of ≤ 0.05 were regarded as statistically significant.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials
Supplemental material may be found here: www.landesbioscience.com/journals/virulence/article/26055/
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