

Phylogenetic Analysis of the Non-structural (NS) Gene of Influenza

A Viruses Isolated in Kazakhstan in 2002-2009*

Andrey Bogoyavlenskiy^{1**}, Vladimir Berezin¹, Alexey Prilipov², Ilya Korotetskiy¹,
Irina Zaitseva¹, Aydyn Kydyrmanov¹, Kobey Karamedin¹, Nailya Ishmukhametova¹,
Saule Asanova¹, Marat Sayatov¹ and Kainar Zhumatov¹

(1. Institute of Microbiology & Virology, 103, Bogenbay batyr Str, Almaty 050010, Kazakhstan; 2. Ivanovsky Institute of Virology, 16, Gamaleya str, Moscow 123098, Russia)

Abstract: Although the important role of the non-structural (*NSI* and *NEP*) gene of influenza A in virulence of the virus is well established, our knowledge about the extent of variation in the *NS* gene pool of influenza A viruses in their natural reservoirs in Kazakhstan is incomplete. 17 influenza A viruses of different subtypes were studied in this paper. Seven types of haemagglutinin and five different neuraminidase subtypes in eight combinations were found among the isolated viruses. A comparison of nucleotide sequences of isolated viruses revealed a substantial number of silent mutations, which results in high degree of homology in amino acid sequences. By phylogenetic analysis it was shown that two distinct gene pools, corresponding to both NS allele A with 5 Clades and B, were present at the same time in Kazakhstan. The degree of variation within the alleles was very low. In our study allele A viruses had a maximum of 5% amino acid divergence in Clade while allele B viruses had only 4% amino acid divergence.

Key words: Influenza; Nonstructural gene; Phylogenetic analysis

Influenza A viruses are enveloped viruses within the family Orthomyxoviridae and are further classified into subtypes depending upon their surface glycoproteins, haemagglutinin (*HA*) and neuraminidase (*NA*). They contain a single-stranded, negative sense, segmented

RNA genome consisting of eight segments of viral RNA (vRNA), which encode 11 known proteins^[25]. Several viral gene products of influenza A virus are known to contribute to the host range restriction and virulence of the virus. The receptor binding efficiency and high cleavability of the haemagglutinin (*HA*) glycoprotein can influence viral entry and lethal outcome of infection^[6, 11, 19, 28]. The PB2 polypeptide, encoded by segment 1, confers a strong influence on host range, and residue 627 is a major, although not the sole, determinant of host specificity in PB2^[1, 31, 36].

Received: 2011-06-28, Accepted: 2011-10-21

* Foundation items: USDA-ISTC(The International Science and Technology Center) partner project #K-747p; Institute of Microbiology and Virology project #0103KZO0126 and #0106KZO0581.

** Corresponding author.

Phone/Fax: +7-727-291-8496, E-mail: anpav_63@mail.ru

The other polypeptides in the viral replication complex, PB1, PA and NP, also influence host range, and compatibility between these four polypeptides is important. There are four major lineages of the PB1 gene (human, swine, equine, avian), five for the PA gene (human, swine, equine, gull, all other birds) and five for the NP gene (human and classic swine, Equine/Prague/56, recent equine, gull, other avian strains)^[8].

The *NS* gene of influenza A viruses encodes an mRNA transcript that is alternatively spliced to express two proteins^[12,13]. Translation of the unspliced mRNA encodes a 26 kDa NS1 protein which shares the same ten amino acids from the initiation codon at the N-terminal of the protein with a 14 kDa nuclear export protein (NEP, formerly called NS2) which is translated from spliced mRNA^[9]. Depending on the virus strain, NS1 consists of 124-237 amino acids in length and is expressed exclusively in infected cells.

The NS1 protein contains two functional domains: the N-terminal RNA-binding domain (residues 1-73) and the C terminal effector domain (residues 73-237)^[26].

It has been suggested that the N-terminal RNA binding domain of the NS1 protein has regulatory activities that are important to prevent interferon mediated antiviral responses. Binding of NS1 protein to both single- and double-stranded RNA might: (a) inhibit activation of interferon induced protein kinase PKR^[17], (b) prevent activation of the 2'-5'oligoadenylate synthetase, which is essential for activation of ribonuclease L (RNase L) system^[21], (c) inhibit the activation of IRF-3 and NF- κ B, key regulators of IFN α and β gene expression, by interfering with the retinoic acid-inducible gene I (RIG-I)^[20, 32, 35] and (d) produce suppression of RNA interfering system, by

binding to small interfering RNAs^[14, 23]. Earlier studies had indicated the existence of important amino acid sequence motifs for the function of NS1 protein. Functional analysis implies that amino acids at the N-terminal RNA-binding domain of NS1 were implicated in this function. The arginine at position 38 and the lysine at position 41 contribute to this interaction^[7]. The N-terminal residues 81-113 of NS1 protein can also bind to eukaryotic translation initiation factor 4GI (eIF4GI), the large subunit of the cap-binding complex eIF4F^[26].

The effector domain of the NS1 protein has been associated with regulation of gene expression of the infected cell^[7]. It has been shown that the effector domain of NS1 protein: (a) inhibits 3'-end processing of cellular pre-mRNA by specifically interacting with the 30 kDa subunit of the cleavage and polyadenylation specific factor (CPSF)^[7]. This function is mediated by two distinct domains; one located around residue 186^[13, 21] and the other one around residue 103 and 106^[7, 26], (b) prevent transport of cellular mRNA to cytoplasm by interaction with poly (A) – binding protein II (PABII)^[7]. Amino acids 215 to 237 have been identified as the binding site for PABII^[7].

The NEP consists of 121 amino acids^[14] which, in association with the matrix protein 1 (M1), interacts with cellular export factor (CEF1) and mediates the nuclear export of viral ribonucleoprotein complexes^[23] by connecting the cellular export machinery with vRNPs^[7].

Our knowledge about the *NS* gene pool of influenza A viruses in their natural reservoirs in Kazakhstan is incomplete. Therefore, in this study we analyzed in detail the *NS* gene sequences of 17 influenza A viruses, isolated in Kazakhstan between 2002-2009 in order to

gain more detailed knowledge about the genetic variation of influenza A viruses in their natural hosts.

MATERIALS AND METHODS

Virus isolation and characterization

With the exception of samples A/Almati/01/2009 (from the Department of Molecular Virology, State Research Center of Virology and Biotechnology 'Vector', Koltsovo, Novosibirsk Region, Russia), A/chicken/Astana/6/2005 and A/domestic goose/Pavlodar/1/2005 (from the Institute for Biological Safety Problems, Gvardeiskiy, Zhambyl Oblast, Kazakhstan). All virus isolation was performed in the Laboratory of Ecology Viruses at the Institute of Microbiology and Virology, Almaty in Kazakhstan. Samples were thawed, mixed with an equal volume of phosphate buffered saline containing antibiotics (penicillin 2000 U/mL, streptomycin 2 mg/mL and gentamicin 50 µg/mL), incubated for 20 min in room temperature, and centrifuged at 1 500 r/min for 15 min. The supernatant (0.2 mL/egg) was inoculated into the allantoic cavity of 9-days old embryonated hens' eggs as described in European Union Council Directive 92/40/EEC^[4]. Embryonic death within the first 24 hours of incubation was considered as non-specific and these eggs were discarded. After incubation at 37 °C for 3 days the allantoic fluid was harvested and tested by haemagglutination (HA) assay as describe in European Union Council Directive 92/40/EEC. In the cases where no influenza A virus was detected in the initial virus isolation attempt, the allantoic fluid was passaged twice in embryonated hens eggs. The number of virus passages in embryonated eggs was limited to a maximum of two, to limit laboratory manipulation. A sample was considered negative when

the second passage HA test was negative. The frequency of virus isolation was at least 15-20 percent of the samples. The subtypes of the virus isolates were determined by conventional haemagglutination inhibition (HI) test and neuraminidase inhibition (NI) test, as describe in European Union Council Directive 92/40/EEC^[4].

RNA extraction and PCR with NS1 gene specific primers

RNA was extracted from infective allantoic fluid using an RNeasy Mini Kit (QIAGEN, GmbH, Germany) according to the manufacturer's instructions. The RNA was converted to full-length cDNA using reverse transcriptase by incubation at 42°C for 60 min followed by inactivation of the enzyme at 95°C for 5 min.

PCR amplification with NS gene specific primers (Fw primer: 5'-AGCAAAGCAGGGTGACAAAG-3', Rev primer 5'-AGTAGAAACAAGGGTGTTTTTAT-3') was performed to amplify the product containing the full length NS gene. PCR reactions were carried out in a thermal cycler at 95°C for 2 min, then cycled 35 times between 95°C 20 sec, 58°C for 60 sec and elongation at 72°C for 90 sec and were finally kept at 8°C until later use.

Phylogenetic and sequence analysis

Sequences of the purified PCR products were determined using gene specific primers and BigDye Terminator version 3.1 chemistry (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Reactions were run on an ABI310TM DNA analyzer (Applied Biosystems). Sequencing was performed at least twice in each direction. After sequencing, assembly of sequences, removal of low quality sequence data, nucleotide sequence translation into protein sequence, additional multiple sequence

alignments and processing were performed with the BioEdit software version 7.0.4.1 with an engine based on the Clustal W version 1.83 algorithm. Phylogenetic analysis, based on complete gene nucleotide sequences, were conducted with the Molecular Evolutionary Genetics Analysis (*MEGA*, version 4.0) software package using neighbour joining tree inference analysis with the Tamura-Nei γ -model, with 1000 bootstrap replications to assign confidence levels to branches^[5, 27, 34, 35].

NS sequences obtained from GenBank

The NS gene was analyzed both with only the Kazakhstan influenza isolates and also with virus genes selected to represent global poultry and mammalian origin isolates.

The NS gene sequences of 58 additional influenza A viruses, reported between year 1934 to 2009, obtained from GenBank were used in phylogenetic studies^[22]. Strains were selected by randomly but from

different clades of the NS gene.

Nucleotide sequence accession numbers

The nucleotide sequence data obtained in this study has been submitted to the GenBank database accession numbers are listed in Table 1.

RESULTS

Avian influenza Prevalence

In our study many different influenza A virus subtypes were found to circulate at the same time, in the same geographic region in the Kazakhstan. This finding most likely indicates the existence of a large reservoir of different influenza A viruses in Kazakhstan. Seven types of haemagglutinin and five different neuraminidase subtypes in eight combinations have been isolated in the same country (Table 1).

Phylogenetic analysis

We analyzed the NS gene sequences of 17 influenza A viruses isolated in Kazakhstan together with a selected

Table 1. Influenza A virus isolates collected in Kazakhstan between 2002 and 2009

Viruses	Accession	Allele
A/garganey/Korgalzhyn/865/2004(H3N6)	FJ434368	A
A/herring gull/Atyrau/2216/2007(H16N3)	GU953285	A
A/great black headed gull/Atyrau/2966/2008(H13N6)	GU953281	A
A/herring gull/Atyrau/280/2002(H13N6)	GU953277	A
A/turkey/Almaty/535/2004(H11N9)	GU953273	A
A/tufted duck/Aktau/1456/2006(H4N6)	GU953269	A
A/equine/Almaty/26/2007(H3N8)	GU953265	A
A/greylag goose/Korgalzhyn/1867/2006(H3N6)	GU953251	A
A/common tern/Korgalzhyn/847/2004(H3N6)	FJ434367	A
A/little grebe/Alakol/791/2004(H4N6)	FJ434366	A
A/coot/Alakol/781/2004(H4N6)	FJ434365	A
A/chicken/Astana/6/2005(H5N1)	FJ390032	A
A/domestic goose/Pavlodar/1/2005(H5N1)	EU213069	A
A/Almati/01/2009(H1N1)	GU367336	A
A/teal/Korgalzhyn/1797/2006(H3N8)	GU953261	B
A/garganey/South Kazakhstan/8048/2008(H3N8)	GU953254	B
A/mute swan/Aktau/1460/2006(H5N1)	FJ434376	B

number of isolates, collected between 1934 to 2009, and previously published in the GenBank^[2].

Analysis of the phylogenetic relationships among the NS genes reported in this study clearly shows that two distinct gene pools, corresponding to both NS allele A and B (Fig. 1, 2)^[18], were presented at the same time in the same geographic location in Kazakhstan. Out of 17 isolated viruses 14 (82%) belong to allele A, while three (18%) to allele B. Allele B viruses appear to be less common in natural host species than allele A, comprising only about 18% of the isolates sequenced in this study. The prevalence rates of allele B viruses in North America are much higher than what we have seen in Kazakhstan (30% in North America^[29] versus 18% in Kazakhstan). In Asia the figure is 15 percent, including all viruses of avian origin^[37]. The differences in function, if any, between

allele A and allele B have not been defined, but it has appeared that allele B viruses don't belong to viruses of mammal. All viruses from mammalian species belong to allele A, with only two exceptions, one previously reported virus of equine origin (A/equine/Jilin/1/1989/H3N8) and one virus strain of human origin (A/New York/107/2003 (H7N2)). However, both these viruses are believed to be a direct transmission from avian species^[25]. An earlier study reported the B allele was subdivided into two groups, comprising the American avian group and the Eurasian avian-equine group which were distinguished by synonymous substitutions in the 21LLSMRDMC28 peptide of the NS1 protein^[30]. Thus, it would be very interesting to be able to pinpoint possible differences in function between the NS1 and NEP proteins from allele A and B.

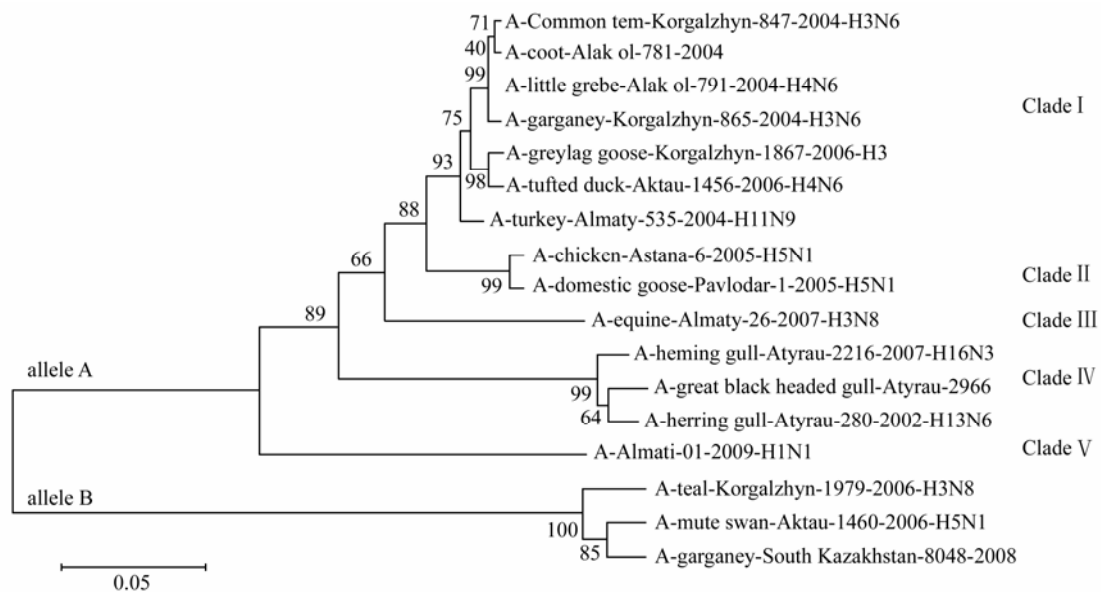


Fig. 1. Evolutionary relationships of 17 influenza strains isolated in Kazakhstan in 2002-2009. The evolutionary history was inferred using the Neighbor-Joining method^[27]. The bootstrap consensus tree inferred from 1000 replicates^[27] is taken to represent the evolutionary history of the taxa analyzed^[27]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches^[27]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Full length of NS genes was used to analyze in figure 1. Phylogenetic analyses were conducted in MEGA4^[34].

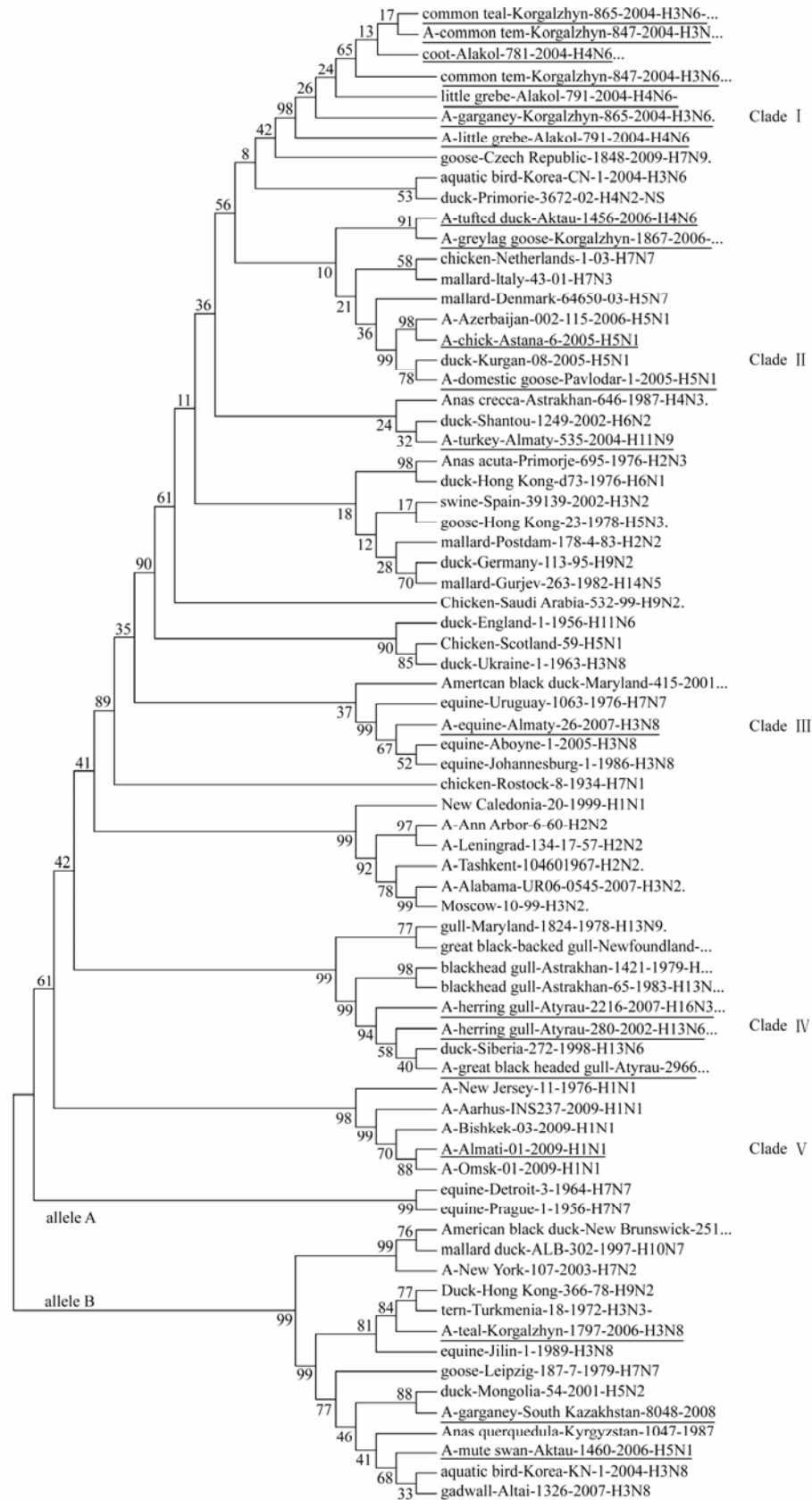


Fig. 2. Evolutionary relationships of 75 influenza strains isolated in 1934-2009. The evolutionary history was inferred using the Neighbor-Joining method [27]. The bootstrap consensus tree inferred from 1000 replicates [27] is taken to represent the evolutionary history of the taxa analyzed [27]. Full length of NS genes was used to analyze in figure 2. Phylogenetic analyses were conducted in MEGA4 [34]. The test strains of influenza are underlined.

Phylogenetic analysis among isolates in allele A revealed five separate clades (Fig 1, 2). Clade I consist of seven isolates divided into three sub clades. Clade II is encompassing two isolates. Clade III and V consists of one isolate. Finally, three isolates formed Clade IV. When co-analyzed with other viruses isolated from different hosts and geographic regions the isolates were grouped the same way: Clade I is encompassing American and Eurasian avian isolates. Clade II consists of pathogenic avian-human group H5N1 viruses, Clade III- represents a group of avian-equine isolates, Clade IV is a unique gull isolate group and Clade V is a human-swine group of viruses. The viruses detected in poultry and in wild birds, grouped closely to each other in both alleles.

Molecular characterization

To further investigate the evolutionary stasis of the *NS* gene, we analyzed the nucleotide and protein sequences of NS1 and NEP of the isolated viruses. Each of the *NS* genes consisted of 689 nucleotides (27-717).

The nucleotide sequences of isolated viruses were compared for similarity. The A/tern/Turkmenia/18/1972 (H3N3) which represents the earliest isolate from wild bird reservoirs, was used as a baseline for allele B viruses. Fifty three nucleotide substitutions were found among the viruses in allele B compared to the reference strain. Of these, forty eight were transitions; 29 were pyrimidine and 19 were purine transitions and five substitutions were results of transversion. Fourteen of these substitutions resulted in amino acid changes in the NS1 protein.

The A/chicken/Rostock/34/ (H7N1) strain represent the earliest isolate from poultry reservoirs and was used as a baseline for allele A viruses. 352 nucleotide

substitutions were found among viruses in allele A compared to this reference strain. 83 of these substitutions resulted in amino acid changes in NS1 protein. Thus, compared to allele A, the degree of variation within the allele B or Clades is very low. A comparison of nucleotide sequences of all isolated viruses revealed a substantial number of silent mutations, which results in high degree of homology in protein sequences.

Analysis of the nucleotide sequence variations demonstrated that nucleotide changes are almost uniformly distributed across the whole gene with only one relatively conserved site at the 3' end of the nucleotide sequence.

Two major functional domains have been predicted for the NS1 protein, the N-terminal RNA-binding domain (residues 1–73) and the C-terminal effector domain (residues 73–237)^[12]. The arginine at position 38 and the Lysine at position 41 contribute to both dsRNA binding activity and interferon antagonist activity of the NS1 protein^[35]. The NS1 gene of all studied isolates includes R38 and K41. The substitution at amino acid position 41 appear more frequently in human isolates of subtypes H1N2 and H3N2 and swine isolates of subtypes H3N2, while the K41 seem to be much more conserved in avian and equine isolates. The majority of human H1N2 and H3N2 viruses contain substitution K41R. No viruses sequenced in this study contained glutamic acid at position 92 of the NS1 protein. Overall, the substitution of Glu92 is extremely rare, and the importance for the virulence in species other than pigs is unclear.

It has been suggested that the amino acid at position 149 of the NS1 protein of HPAI-H5N1 affects the

ability of the virus to antagonize the induction of IFN α/β in chicken embryo fibroblasts^[16]. All Kazakhstani isolates sequenced in this study possessed the amino acid Ala149 in their NS1 protein and have this proposed virulence hallmark of NS1.

The NS1 protein interaction with cleavage and polyadenylation specificity factor (CPSF) inhibits 3'-end processing of cellular pre-mRNA^[3, 15, 22]. This function is mediated by two distinct domains; one around residue 186^[15] and the other one around residue 103 and 106^[10]. All isolates sequenced in this study possessed amino acids Glu186 (or Lys186 for equine virus), Phe/Tyr103 and Met106 in their NS1 protein. It was proposed earlier^[24] that NS1 has a PDZ binding motif at the very end of the protein. PDZ domains are protein-interacting domains present once or multiple times within certain proteins and these domains are involved in the cell signaling, assembly of large protein complexes and intracellular trafficking. It has also been shown that there are typical human, avian, equine and swine motifs.

The most commonly seen avian motif ESEV has been shown to bind to several PDZ domains in human proteins, while the most common human motif RSKV binds very few. All the nonpathogenic avian viruses isolated in Kazakhstan possessed the typical avian ESEV amino acid sequence at the C-terminal end of the NS1 protein. Pathogenic viruses H5N1 have ESKV, equine virus has KPEI and human- GTEI. However, viruses from Asia have other slight variants such as EPEV and GPEV. The EPEV motif appears in both avian as well as swine, human and equine viruses. It is therefore possible that this motif of NS1 is important for the adaptation of influenza into a new host. The exact functional relevance of this remains unclear at

the moment.

All Kazakhstan isolates sequenced in this study possessed two variants in the sequence of the NEP, at position 14, which are particularly important for the attenuation of replication of the avian influenza viruses in human.

DISCUSSION

Our surveillance study indicates existence of a large reservoir of different influenza A viruses in Kazakhstan. This is due to the large territory of Kazakhstan, through which passes the three main migration ways for migratory birds in Eurasia. Seven subtypes of haemagglutinin and five different neuraminidase subtypes in eight combinations were found among the isolated viruses.

Finally, to our knowledge, this is the first study providing a comprehensive analysis of the NS gene of avian influenza in its natural reservoir in Kazakhstan. Our findings improve the present understanding of NS gene pool of avian influenza viruses and should help in improving understanding of gene function in natural hosts. Allele B viruses appear to be less common in natural host species than allele A, comprising only about 18% of the isolates sequenced in this study.

Despite the high level of subtype variation among studied viruses, the nucleotide sequences of NS gene of these viruses showed a substantial number of silent mutations, which results in high degree of homology in protein sequences.

Acknowledgements

The research was supported by USDA-ISTC (The International Science and Technology Center) partner

project #K-747p; Institute of Microbiology and Virology project #0103KZO0126 and #0106KZ00581.

References

1. **Almond J W.** 1977. A single gene determines the host range of influenza virus. *Nature*, 270:617-618.
2. **Bao Y P, Bolotov, Dernovoy D, et al.** 2008. The Influenza Virus Resource at the National Center for Biotechnology Information *J. Virol*, 82(2):596-601.
3. **Chen Z, Li Y, Krug R M.** 1999. Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3'-end processing machinery. *EMBO J*, 18:2273-2283.
4. European Union Council Directive 92/40/EEC of 19 May 1992 introducing Community measures for the control of avian influenza *Official Journal L* 167, 22/06/1992. 0001-0016.
5. **Felsenstein J.** 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39:783-791.
6. **Gagneux P, Cheriyan M, Hurtado-Ziola N, et al.** 2003. Human-specific regulation of alpha 2-6-linked sialic acids. *J Biol Chem*, 278:48245-48250.
7. **Hale B G, Randall R E, Ortin J, et al.** 2008. The multifunctional NS1 protein of influenza A Viruses. *J Gen Virol*, 89: 2359-2376.
8. **Hatta M, Gao P, Halfmann P, et al.** 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science*, 293:1840-1842.
9. **Inglis S C, Barrett T, Brown C M, et al.** 1979. The Smallest Genome RNA Segment of Influenza Virus Contains Two Genes that May Overlap. *Proc Natl Acad Sci USA*, 76:3790-3794.
10. **Kochs G, Garcia-Sastre A, Martinez-Sobrido L.** 2007. Multiple AntiInterferon Actions of the Influenza A Virus NS1 Protein. *J Virol*, 81:7011-7021.
11. **Kuiken T, Holmes E C, McCauley J, et al.** 2006. Host species barriers to influenza virus infections. *Science*, 312:394-397.
12. **Krug R M, Yuan W, Noah D L, et al.** 2003. Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. *Virology*, 309:181-189.
13. **Lamb R A, Choppin P W.** 1979. Segment 8 of the Influenza Virus Genome is Unique in Coding for Two Polypeptides. *Proc Natl Acad Sci USA*, 76:4908-4912.
14. **Lamb R A, Lai C J.** 1980. Sequence of interrupted and uninterrupted mRNAs and cloned DNA coding for the two overlapping nonstructural proteins of influenza virus. *Cell*, 21:475-485.
15. **Li Y, Chen Z Y, Wang W, et al.** 2001. The 3'-end-processing factor CPSF is required for the splicing of single-intron pre-mRNAs in vivo. *RNA*, 7:920-931.
16. **Li Z, Jiang Y, Jiao P, et al.** 2006. The NS1 Gene Contributes to the Virulence of H5N1 Avian Influenza Viruses. *J Virol*, 80:11115-11123.
17. **Li S, Min J Y, Krug R M, et al.** 2006. Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. *Virology*, 349:13-21.
18. **Ludwig S, Schultz U, Mandler J, et al.** 1991. Phylogenetic relationship of the nonstructural (NS) genes of influenza A viruses. *Virology*, 183:566-577.
19. **Matrosovich M, Tuzikov A, Bovin N, et al.** 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. *J Virol*, 74:8502-8512.
20. **Mibayashi M, Martinez-Sobrido L, Loo Y M, et al.** 2007. Inhibition of Retinoic Acid-Inducible Gene IMediated Induction of Beta Interferon by the NS1 Protein of Influenza A Virus. *J Virol*, 81:514-524.
21. **Min J Y, Krug R M.** 2006. The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway. *Proc Natl Acad Sci USA*, 103:7100-7105.
22. **Nemeroff M E, Barabino S M L, Li Y, et al.** 1998. Influenza Virus NS1 Protein Interacts with the Cellular 30 kDa Subunit of CPSF and Inhibits 3' End Formation of Cellular PremRNAs. *Mol Cell*, 1:991-1000.
23. **Neumann G, Hughes M T, Yoshihiro K.** 2000. Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. *EMBO J*, 19:6751-6758.
24. **Obenauer J C, Denson J, Mehta P K, et al.** 2006. Large-Scale Sequence Analysis of Avian Influenza Isolates *Science*. 311:1576-1580.
25. **Palese P, Shaw M L.** 2007. Orthomyxoviridae: the viruses and their replication. In: *Fields Virology*, 5th edn, (D. M. Knipe & P. M. Howley,eds.). Philadelphia: Lippincott Williams & Wilkins. p1647-1689.

26. **Qian X Y, Alonso-Caplen F, Krug R M.** 1994. Two functional domains of the influenza virus NS1 protein are required for regulation of nuclear export of mRNA. *J Virol*, 68:2433-2411.
27. **Saitou N, Nei M.** 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol.* 4:406-425.
28. **Shinya K, Ebina M, Yamada S, et al.** 2006. Avian flu: influenza virus receptors in the human airway. *Nature.* 440:435-436.
29. **Spackman E, Slemons R D, Winker K, et al.** 2005. Phylogenetic analyses of type A influenza genes in natural reservoir species in North America reveals genetic variation. *Virus Res*, 114:89-100.
30. **Suarez D L, Perdue M L.** 1998. Multiple alignment comparison of the non-structural genes of influenza A viruses *Virus Res.* 54:59-69.
31. **Subbarao E K, London W, Murphy B R.** 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. *J Virol*, 67:1761-1764.
32. **Talon J, Horvath C M, Polley R, et al.** 2000. Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. *J Virol*, 74:7989-7996.
33. **Tamura K, Dudley J, Nei M, et al.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol.* 24:1596-1599.
34. **Tamura K, Nei M, Kumar S.** 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci USA*, 101:11030-11035.
35. **Wang W, Riedel K, Lynch P, et al.** 1999. RNA binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids. *RNA*, 5:195-205.
36. **Yao Y, Mingay L J, McCauley J W, et al.** 2001. Sequences in influenza A virus PB2 protein that determine productive infection for an avian influenza virus in mouse and human cell lines. *J Virol*, 75:5410-5415.
37. **Zohari S, Gyarmati P, Ejdersund A, et al.** 2008. Phylogenetic analysis of the non-structural (NS) gene of influenza A viruses isolated from mallards in Northern Europe in 2005 *Virol J*, 5:1-13.