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Short Communication

Adaptive changes in influenza A virus H7N2

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ABSTRACT

The North American low pathogenic avian influenza A virus H7N2, that lacks the 220-loop in the hemagglutinin (HA), possesses dual receptor specificity with a strong affinity for the avian and a weak affinity for the human sialic acid receptors. To estimate the possibility of interspecies transmission by H7N2 viruses, we have serially passaged a chicken virus strain, through mouse lungs, to adapt the virus for mice. Once mouse adapted (MA), the original apathogenic virus became virulent with 13 amino acid substitutions in five viral proteins (PB2, HA, NA, NS1, NEP). The four mutations in the HA1 chain did not significantly change receptor specificity, but elevated the HA thermostability and the pH value of HA activation. NS1 was the most modified protein, with six amino acid substitutions. All substitutions occurred at sites that were polymorphous in the original wild strain. The minor alternative amino acids of the original virus NS1 remained in the MA variant. The MA influenza virus also acquired the adaptive mutation E627K in PB2. In addition to E627K in PB2, four other mutations (N73T, G171A, F214L in NS1, and E14Q in NEP) can be considered to be adaptive because they are common among viruses H7N2 isolated from mammals (human, cat, and mouse).

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KEYWORDS: avian influenza virus H7N2, adaptive mutations, host range change, interspecies transmission.

INTRODUCTION

Avian influenza A viruses (AIVs) of the H7 subtype represent a significant threat to commercial poultry due to the disease they cause or the trade restrictions enforced when the viruses are detected at farms. Additionally, human infections with H7 avian influenza viruses have been reported several times around the world, with varying severity in clinical outcomes [1]. The H7 AIVs circulating in the Western and Eastern Hemispheres are phylogenetically different. The most severe and prolonged epidemics, caused by both low- and highly pathogenic H7N9 AIVs, were reported in China between 2013-2017. In total, 1568 humans were infected, of which 615 died [2, 3]. In contrast, since 1959, only 12 cases of human infection with H7 viruses have been reported on the North American continent (Canada, Mexico, USA) [1]. Three major genetic clusters of HA/H7 genes were identified in the North American lineage [4]. Cluster I includes viruses that were isolated from waterfowl and domestic birds in 1969-1993. Cluster II consists mainly of viruses that circulated in domestic poultry from 1994-2006. Cluster III combines AIVs of wild waterfowl and domestic birds. This cluster emerged in 2000 and is ongoing [4, 5]. Some viruses of cluster III have caused

outbreaks in poultry. Phylogenetic relations, genetic changes, and mechanisms in which these wild bird H7 viruses of cluster III become highly pathogenic for poultry are thoroughly outlined in [5].

Cluster II is comprised of AIVs from subtype H7N2 that were isolated from live bird markets in northeastern United States and from commercial poultry farms in four US states (Maryland, Pennsylvania, North Carolina, and Virginia). This cluster is further divided into two clades: II-1 and II-2. Clade II-1 consists of viral isolates from 1994–1996 and clade II-2 of isolates from 1996–2006. The main molecular feature of clade II-2 is a deletion of eight amino acids at positions 221-228 (in H3 numbering) located in the 220-loop of HA. In 2003, a single strain A/New York/107/2003(H7N2), belonging to clade II-2, was isolated from a human with suspected exposure to poultry [4]. Much later, in 2016-2017, an outbreak of H7N2 influenza A virus infected approximately 500 domestic cats in a New York animal shelter. A veterinarian, who was treating the infected cats, also then displayed an influenza-like illness. Complete genome sequencing of the feline and human viral samples found that both viruses had the 220-loop deletion in HA and were closely related to low pathogenic avian influenza H7N2 viruses that were circulating in US during the early 2000s [6-8].

In this work, we sought to discover the adaptive mutations of a North American low pathogenic avian influenza virus H7N2 to mammals by using a mouse model.

MATERIALS AND METHODS

Low pathogenic avian influenza A virus A/chicken/NJ/294598-12/2004 of subtype H7N2 (ch/NJ) was isolated in New Jersey, USA, and kindly provided for study by Dr. Alexander I. Klimov (Centers for Disease Control and Prevention, Atlanta, GA 30333, USA). This original strain was maintained in 10-day-old embryonated chicken eggs.

White outbred mice were intranasally infected with undiluted virus containing allantois fluid. After 48 h, mice were humanely euthanized, and lung homogenate was prepared for subsequent intranasal infection. After 10 serial passages, the virus had become pathogenic and lethal for the mice. The mouse adapted virus variant was cloned once by limited dilution in embryonated chicken eggs and named A/chicken/NJ/294598-12MA/2004 (MA/NJ).

Receptor-binding properties of influenza viruses were determined according to [9].

The hemagglutination test, detection of the viral 50% egg infective dose (EID_{50}), and the 50% mouse lethal dose (LD_{50}), HA thermostability, estimation of HA activation pH values by hemolysis assay, and Sanger nucleotide sequencing were performed as described earlier [10]. Nucleotide sequences of all eight segments of original strain coincided with those of similar related strain (GenBank Accession Numbers MN400388 - MN400395).

RESULTS AND DISCUSSION

Phenotypic properties

The AIV A/chicken/NJ/294598-12C/2004 (H7N2) was originally apathogenic for mice that were intranasally infected with virus-containing allantois fluid at doses of $10^{8,6}$ to $10^{9,6}$ EID_{50}. In contrast, the adapted MA/NJ variant was 100% lethal when a dose of $10^{4,7}$ EID_{50} was given.

In addition to pathogenicity, the original H7N2 virus and the MA/NJ variant had differences in other phenotypic features. The MA/NJ strain possessed an average 2.8 ºC increase in thermal stability of HA compared to the original avian variant. Furthermore, the HA activation pH value increased by 0.4 units in the MA/NJ variant. (Table 1). An increase of these values is typically seen in viruses with elevated pathogenicity.

Receptor binding specificity was tested using a binding assay of viruses to sialyloligosaccharides of cellular receptor analogs for the avian (3'SLN) or human type (6`SLN) conjugated with polyacrylamide [9]. According to our previous study, the original strain (ch/NJ) possessed maximal binding to sulfated sialyloligosaccharides Su-3'SLN, high affinity for avian-type receptors 3'SLN, and moderate binding affinity for human-type receptor 6'SLN [11]. In this work, the MA/NJ variant retained dual receptor binding specificity to 3'SLN and 6`SLN, although its affinity for 6`SLN became weaker than ch/NJ (Table 1).

Table 1. Phenotypic properties of the original (ch/NJ) and mouse-adapted (MA/NJ) variants of the A/chicken/NJ/294598-12C/2004 (H7N2) virus.

Virus strain	Pathogenicity[1]	Temperature of HA inactivation, (ºC±0.2ºC)	HA activation pH value	Affinity for receptor analogues, K_{diss}, nm SA[2]		
				Fet-HRP	3`SLN	6`SLN
ch/NJ	>9.6 (nonpathog.)	62.3	5.2±0.2	200±100	100±50	200±50
MA/NJ	4.0±0.2	65.1	5.6±0.2	200±100	100±50	500±50

Notes. The average values of three independent experiments are presented.
[1]Pathogenicity for mice is represented as $\log_{10}$ of EID_{50} in one unit of LD_{50}. A lower value corresponds to a higher pathogenicity.
[2]The results of titration with peroxidase-labeled fetuin conjugate (Fet-HRP) and biotinylated polymers 3'SLN (Neu5Acα2-3Galβ1-4GlcNAcβ) and 6'SLN (6'SLN - Neu5Acα2-6Galβ1-4GlcNAcβ) are presented as a dissociation constant expressed in sialic acid nanomoles. A higher value corresponds to a lower affinity for cellular receptor analogues.

Table 2. Amino acid differences in the original (ch/NJ) and mouse-adapted (MA/NJ) variants of the A/chicken/New Jersey/294598-12C/2004 (H7N2) virus.

Protein	Position of a.a.	ch/NJ, a.a	MA/NJ, a.a.	Location (Function)	Reference
PB2	627	E	K	Domain 627 (contact with host cellular factors; host species specificity; determinant of pathogenicity)	[17-20]
HA	125/133[1]	F	F<**L**	Antigenic site A	[21, 22]
HA	156/164	N<**D**[2]	N	Antigenic site B	[21, 22]
HA	198/207	G	E	Antigenic site B	[21, 22]
HA	328/330	K	T	HA cleavage site	[12]
NA	127	K	N	Head domain, epitope	[13]
NS1	73	T<**N**	T	RNA binding domain (binding several RNA species, including dsRNA. Also mediates interactions with some host transport proteins)	[14, 15]
NS1	114	G<**S**	G	Effector domain (domain mediates interactions with several host proteins and may stabilize the N-terminal RNA-binding domain)	[14, 15]
NS1	118	R<**K**	R		[14, 15]
NS1	171	A<**G**	A		[14, 15]
NS1	214	L<**F**	L	C-terminal domain	[14, 15]
NS1	224	R≤**G**	R		[14, 15]
NEP	14	Q<**E**	Q	Nuclear export signal	[16]

[1]The numbering of HA corresponds to H3/H7. Numbering is given for the mature HA/H3 protein of strain A/Aichi/2/68, and for HA/H7 according to the sequence ACF25499 (GenBank).
[2]The ratio of alternative amino acids (a.a.). The predominant a.a. is in bold font.

Molecular changes

Complete genomes of both virus variants were sequenced to determine changes at the molecular level. Amino acid mutations occurred in five viral proteins in the MA/NJ variant: PB2, HA, NA, NS1, and NEP (Table 2).

Comparison of the amino acid sequences of HA for ch/NJ and MA/NJ revealed four substitutions (Table 2). Although these substitutions did not significantly change receptor specificity, they may have affected the HA activation pH and the thermal stability of HA and the virion. The HA

activation pH and the thermal stability of HA are important for viral genome penetration into host cells. This is due to the process of attachment to the cell surface and subsequent conformational changes of HA triggered by the decreasing pH in the endosome, resulting in the uncoating of the viral genome in the cytoplasm.

Three HA substitutions (F125/133L, D156/164N, G198/207E in numbering H3/H7) were located in antigenic sites and one substitution (K328/330T) was situated in the -2 position of the HA cleavage site (between HA1 and HA2 chains). This was consistent with the low pathogenic phenotype (EKPK**T**R↓G). A similar amino acid (-2T) was present in North American H7N2 viruses of clade II-1, but it is unlikely that this mutation changed viral pathogenicity [12].

The N1 neuraminidase of highly pathogenic avian influenza H5N1 and the N2 of the North American H7N2 viruses contains a deletion of 16 amino acids in the stalk domain (56-71 a.a.)[1]. This deletion is considered to be a genetic marker of poultry-adapted viruses. Substitution K127N (143) is situated on the surface of the head domain in an experimentally determined epitope[2] 114-130 a.a. (130-146) and has a similar structure in both ch/NJ and other reference strains. Moreover, in the 3D structure [13], amino acid residue 127K (143) of one chain contacts residue 450F (466) of an adjacent chain in the homotetrameric NA protein. The substitution of K with N, an exchange of a relatively large residue with a small one, caused the disappearance of its originally positive charge. This may have altered the interaction between the adjacent chains in MA/NJ. This mutation (K127N) in NA of North American H7N2 viruses has not been described before.

Unexpectedly, ch/NJ existed as a heterogenic population, in contrast to MA/NJ. The HA and NS proteins contained multiple polymorphic sites where the ch/NJ quasispecies had different amino acids. Finally, only the minor alternative amino acid of ch/NJ existed in MA/NJ (Table 2). Most of the substitutions occurred in the effector and C-terminal domains of NS1, where motives and signaling regions for multiple cellular host factors are located. Of the NS1 mutations found in the MA/NJ variant, three mutations (N73T, G171A, F214L) were also found in viruses that were isolated from mammals (mouse, cat and human) and belonged to the same North American clade II-2. Taking into account that the NS gene belongs to the type B allele, which is typical in avian influenza viruses compared to mammalian influenza viruses where the NS gene belongs to type A, we can assume that the substitutions found here are related to the adaptation of chicken-derived virus to a mammalian host.

The NS1 protein is necessary for influenza A viruses to evade the innate immune system and replicate effectively in a host. Due to the interaction of NS1 with many cellular factors, it plays an important role in virus-host interactions and in the process of interspecies adaptation [14, 15].

The single mutation E14Q in the NEP protein of MA/NJ occurred in the nuclear export signal motif (12-21, NES) which interacts with cellular transport protein CRM1 and mediates export of viral ribonucleoprotein complexes from the nucleus into the cytoplasm (Table 2) [16]. This is another common mutation in influenza H7N2 viruses isolated from mammals.

E627K in PB2 is a well-known mammalian adaptive mutation [17-20] which appeared during the adaptation of avian influenza virus ch/NJ to mice. It should be of note that this mutation is not present among other North American H7N2 viruses isolated from human or cats [6, 7].

CONCLUSIONS

In this study, we utilized a mouse model to elucidate the adaptive properties of a low pathogenic avian influenza virus belonging to North American H7N2 lineage which lacked the 220-loop in HA. Mutations were found in five viral proteins (PB2, HA, NA, NS1, and NEP) of the resulting mouse-adapted viral variant. In addition to the known adaptive mutation E627K in PB2, analysis suggests that four other mutations (N73T, G171A, F214L in NS1, and E14Q in NEP) can be considered

[1]In parentheses is NA numbering according to reference strain A/Tokyo/3/1967(H2N2), GenBank: AAO46245; PDB ID: 1INH.

[2]Sequence Feature ID: Influenza A_N2_SF110. Online: https://legacy.fludb.org/brc/home.spg?decorator=influenza

adaptive to mammals because they are commonly found among viruses of this clade isolated from mammals. Although the contribution of HA and NA mutations cannot be excluded, the biggest contributor to increased viral pathogenicity is the mammalian adaptive mutation E627K in the polymerase protein PB2.

ETHICS STATEMENT

Studies involving animals were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, Strasbourg, 18 March 1986, and in strict compliance with the rules for carrying out work with the use of experimental animals (Order No. 266 of the Ministry of Health of the Russian Federation dated 6/19/2003).

The study design was approved by the Ethics Committee of the Gamaleya National Research Center for Epidemiology and Microbiology, Ministry of Health of the Russian Federation, Moscow, Russia (Approval № 12 from 28 June 2021).

CONFLICT OF INTEREST STATEMENT

The authors have no commercial or financial interests.

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