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Characterization of highly pathogenic avian influenza (HPAI) A subtype H5N1 strains isolated from an outbreak in poultry and wild birds in Western Siberia, July 2005

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Abstract

Complete genomes of two highly pathogenic avian influenza (HPAI) A (H5N1) strains isolated from wild birds (A/Grebe/Novosibirsk/29/05) and poultry (A/Duck/Novosibirsk/56/05) during an epizootic in 2005 summer in one location in Western Siberia (Novosibirsk region, Russia) were analyzed. These strains had a basic amino acid motif in the hemagglutinin cleavage site characteristic of HPAI influenza. They differed genetically from the H5N1 avian viruses isolated earlier but were closely related in all genes to the H5N1 viruses isolated from wild birds in Qinghai Lake, China, in May 2005. There was slight genetic differences between the two Western Siberian strains (2005) within the PB1, PB2, PA and NP genes (from one to four amino acid substitutions), but both viruses had Lys-627 in PB2 protein, Glu-92 in NS1, Ser-31 in M2 and a 20-mer deletion in the NA gene. Both isolates were sensitive to remantadine, amantadine, ribavirin, arbidol in porcine embryo kidney cell line.

The isolation of low pathogenic avian influenza A (LPAI) subtype H5N2 and H5N3 strains from wild birds in 1991-2001 in different regions of Siberia and Far East of Russia demonstrated that H5 influenza can be routinely found in the wild bird population. These LPAI viruses when transmitted to poultry on rare occasions can mutate to the highly pathogenic form of the virus. It was possible that LPAI influenza A H5 subtype viruses from Northern Eurasia could be precursors of H5N1 HPAI viruses in South East Asia (1-3) and for the H5N1epizootics in 2003 (4-7).

In 2005 July an epizootic outbreak of avian influenza occurred among wild birds and poultry in the territory of Western Siberia, Russia. A brief report describing the isolation of several strains of HPAI H5N1 and deposition of viruses to Russian State Collection of Viruses (RSCV) was presented by (8). Here we describe some of the genetic and some biological properties of two H5N1 avian influenza virus isolates from Great crested grebe (*Podiceps cristatus*) and domestic duck (*Anas platyrhynchos domesticus*).

Materials and methods

We used for study **strains** of HPAI (H5N1) deposited in RSCV, specifically those isolated from a clinically healthy Great crested grebe (*Podiceps cristatus*) A/Grebe/Novosibirsk/29/05 (H5N1) (RSCV reg. N 2372) and from a sick domestic duck (*Anas platyrhynchos domesticus*) A/Duck/Novosibirsk/56/05 (H5N1) (RSCV reg. N 2371) (8). The original swabs from which these strains was isolated were collected on July 28, 2005 in one village on the territory of Chany Lake lowland of Barabinskaya steppe-forest of Novosibirsk region in the limits of the epicenter of epizootic outbreak among poultry starting about July 15, 2005.

Cell cultures used for the examination of avian influenza strain replication potential and cell tropism were embryo pig kidney cell line (PS), green monkey kidney

cell line, clone #6 (Vero-E6), human embryo lung cell line (LEH), hamster kidney cell line (BHK-21), canine kidney cell line (MDCK). Medium 199 was used for PS cell line cultivation, DMEM with glutamine was – for Vero-E6, LEH, BHK-21 and MDCK. Each medium contained 10 % embryo calf serum, 10 mM glutamine and 100 U/ml penicillin and streptomycin. Cell cultures as one day-old monolayers growing in 24-well plastic culture panels were used for virus inoculation.

Antiviral compounds: amantadine hydrochloride ("Olinefarm", Latvia,), rimantadine hydrochloride (AS "Adamantan", Russia), ribavirin (ICN, USA), arbidol ("Masterlek", Russia). The compounds were dissolved in 199 or DMEM media at 1 mg/ml.

Antiviral assay. PS cells were seeded in 96-well culture plates and incubated at 37°C with 5 % CO_2 to reach 90 % confluence. Cells were washed twice with serum-free 199, and residual medium was removed. Each microtiter plate included uninfected control cultures, virus-infected control wells, and virus-infected cultures to which antiviral drugs were added. The cultures were overlaid with 199 containing TPCK-treated trypsin 2.0 µg/ml and twice the concentration of the antiviral drugs being studied (100 µl). After incubation for 30-90 min at 37°C, 100°µl of virus containing fluid (0.1 $TCID_{50}$/cell) was added to all wells excluding the wells used as cell controls. Monolayers were fixed after 18-20 h of incubation at 37°C.

The extent of viral replication was assessed by modified enzyme-linked immunosorbtion assay (ELISA), which detected expression of viral proteins on the surface of infected cells (9). The percent of virus replication inhibition (PVRI) by antiviral drugs was calculated as:

$$PVRI = \left(1 - \frac{OD_T}{OD_C}\right) \cdot 100\% ,$$

where OD_T is the optical density at 450 nm of treated sample, OD_C – the optical density at 450 nm of the virus control sample. The concentrations of the drugs that effectively inhibited virus replication by 50 % were determined by plotting inhibition of virus replication as a function of drug concentration.

Sequencing of influenza A genes was performed using an ABI Prism 3130 (Applied Biosystems, USA) automated sequencer according to manufacturer's recommendations. Analysis of nucleotide and relative amino acid sequences was carried out using the software package DNASTAR (DNASTAR Inc., USA).

Phylogenetic analysis of nucleotide sequences obtained both during presented work or in GenBank database was performed using single-linkage algorithm in the limits of M. Kimura two-parameter model with 1000-multiple resampling (MEGA 3.0).

Results and discussion

The two Western Siberian HPAI (H5N1) strains replicated well in all five cell lines without trypsin with widespread cytopathogenic effect (CPE). The infectious titers varied in different cell lines from 4.0 upto 7.0 $\log_{10}TCID_{50}$/ml, and the hemagglutination titer – from 8 upto 256 (Table 1). The grebe virus had less *in vitro* infection potential compared to the one from domestic duck. Sensitivity of cell lines increased in the order BHK-21 → LEH → Vero-E6 → MDCK → PS for both examined strains, although the PS cell line had been initially used for virus isolation from field samples (8). All cell lines of this study were of mammalian origin, and notably included human embryo lung cells (LEH). The wide spectrum of cell tropism may be explained by virus population heterogeneity, which previously has been already assumed for HPAI / H5N1 variants selecting for new host after the single passage (10-11).

PS cells infected by A/Duck/Novosibirsk/56/05 strain was chosen for the further investigation of the inhibition effect of some commercial antiviral drugs on HPAI (H5N1) virus replication. Rimantadine, amantadine, ribovirin and arbidol effectively inhibited reproduction of HPAI / H5N1 virus with an *in vitro* effective doses from 1.50 upto 9.70 µg/ml (Table 2), whereas all tested compounds were not toxic upto 40 µg/ml (data not presented). Anti-virus action increases in the order of remantadine → amantadine → ribavirin → arbidol in a dose-dependent manner. The susceptibility of A/Duck/Novosibirsk/56/05 to amantadine and rimantadine correlates with its M2 gene genetic sequence, e.g. serine at position 31 of M2 (Table 3) (6, 18).

Although vaccine can be effective at reducing clinical disease for human influenza, it is impractical to prepare vaccines for all possible pandemic strains of avian influenza. So, antiviral drugs are likely to play a major role in reducing the impact of the next pandemic during the initial stages of the outbreak (12-13). The data from the strains examined show these viruses are susceptible to several classes of commonly used influenza antiviral drugs, and would be amenable to anti-viral invention.

All eight segments of genomes of A/Grebe/Novosibirsk/29/05 and A/Duck/Novosibirsk/56/05 have been sequenced and the data is available in GenBank with accession numbers DQ230521-24, DQ232605-10, DQ234073-78. Both the wild bird and duck strains are closely related to each for all eight gene segments (Figure 1) and have several notable features (Table 3) including: 1. a multiple basic amino acid motif PQGERRRKKRGLF in the cleavage site of the HA which is characteristic of the Asian HPAI H5 lineage (3, 14); 2. Lys-627 in PB2 protein, which has been associated with increased virulence in mammal models of disease (15, 16); 3. Glu-92 in NS1 protein, which has been related to increased resistance to the antiviral action of interferons, tumor necrosis α-factor and is associated with increased virulence for pigs (17); 4. Ser-31 in M2 protein associated with rimantadine / amantadine sensitivity whereas Asp-31 mutation as

seen in strains from South East Asia leads to rimantadine / amantadine resistance (6, 18) (see Table 2); 5. a twenty amino acid deletion in the stalk of the NA segment (positions 49-68), that may be associated with adaptation to poultry (5, 19). The last 20-mer deletion is one of the main markers of so called genotype Z viruses that has dominated among epizootic strains from South East Asia as well as strains infecting humans in 2004 (5, 6) (e.g. A/Vietnam/1194/04 (H5N1) on Figure 1). Genotype Z was turned to be different with genotype Z+, isolated in 2002 from wild waterfowls and humans in 2003 (20).

The Western Siberian HPAI (H5N1) strains (July 2005) are phylogenetically similar with A/Qinghai/05 group of strains (Figure 1), which were isolated from wild birds in May of 2005 during epizootic outbreak among wild birds – mainly among Bar-headed geese (*Eulabeia indica*) and Great black-headed gulls (*Larus ichthyaetus*) – at Qinghai Lake in North-Western China. These viruses are HPAI (H5N1) genotype Z, but are different from those that caused human infection in Vietnam and Thailand (4) (e.g. A/Thailand/04 group on Figure 1). This strongly suggests that the route of introduction of HPAI (H5N1) virus from Central and South Eastern Asia into Northern Eurasia is via long-range migration of aquatic and semi-aquatic birds through the so called "Dzhungarian Gate" (8, 21).

The genetic diversity between A/Grebe/Novosibirsk/29/05, A/Duck/Novosibirsk/56/05 and A/Qinghai/05 group of strains is presented in Table 3. The highest difference between A/Grebe/Novosibirsk/29/05 and A/Duck/Novosibirsk/56/05 occurs in PB1 and PB2 proteins (2 and 4 amino acid substitutions, respectively) whereas HA1 subunit, M1, M2, NS1 and NS2 proteins are identical. Amino acid sequences of HA2, PA and PB1 proteins of A/Grebe/Novosibirsk/29/05 strain slightly differ from ones of both A/Duck/Novosibirsk/56/05 and the Qinghai/05 strains. The total of 11 amino acid differences were found between two Western Siberian HPAI (H5N1) strains; between

A/Grebe/Novosibirsk/29/05 and the Qinghai/05 strains the differences is 11; between A/Duck/Novosibirsk/56/05 and Qinghai/05 is 5 (see Table 3). In explaining the close genetic relations between Western Siberian strain HPAI / H5N1 from domestic duck and viruses from Qinghai Lake in 2005, it is necessary to take into account two circumstances. Firstly, according to the field register, Great crested grebe used for A/Grebe/Novosibirsk/29/05 isolation was a juvenile, therefore this bird was linked with the precursor virus variant not directly, but through its parents. Secondly, genotype Z, that includes Western Siberian and Qinghai strains (2005), have increased virulence for poultry where most of the rapid evolution and genetic diversity of these viruses arises [12, 17]. It is unclear if these viruses isolated in Qinghai Lake and Western Siberia form a separate sub-lineage established in wild migrating bird populations or is related to viruses arising from domestic poultry. More genetic analysis of viruses from different geographic regions needs to be carried out to provide the answer to this question.

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Table 1. Spectrum of cell lines sensitive for Western Siberian HPAI / H5N1 strains (2005).

Cell line	Origin of the cell line	Markers of virus replication (without trypsin in culture medium)			
		A/Grebe/Novosibirsk/29/05		A/Duck/Novosibirsk/56/05	
		CPE * titer ($log_{10}TCID_{50}$/ml)	Hemagglutination titer	CPE * titer ($log_{10}TCID_{50}$/ml)	Hemagglutination titer
PS	Embryo pig kidney cell cultures	5.0	64	7.0	256
Vero-E6	Green Monkey kidney cell line, clone #6	4.0	16	5.0	64
LEH	Human embryo lung cell line	2.5	16	4.5	32
BHK-21	Hamster kidney cell line	4.0	8	5.0	32
MDCK	Canine kidney cell line	5.7	64	6.5	256

* CPE – cytopathogenic effect

Table 2. Inhibition of Western Siberian HPAI / H5N1 strain A/duck/Novosibirsk/56/05 replication in PS cell line by antiviral compounds.

Criteria	Concentration (µg/ml)			
	amantadine	**remantadine**	**ribavirin**	**arbidol**
Inhibition of virus replication tested by infection titer measuring *	1.50	1.25	2.50	7.00
Inhibition of antigen expression tested by HIT **	2.00	1.25	2.50	6.50
Inhibition of antigen expression tested by ELISA ***	no data	3.70	4.10	9.70

* Concentration of the compounds inhibiting CPE by 50 % as compared to untreated control, in which CPE was assumed to be 100 %.

** Concentration of the compounds completely restricting hemagglutinin production to culture medium of infected PS cells.

*** Concentration of the compounds reducing ELISA optical density at 450 nm by 50 % as compared to untreated control, in which CPE was assumed to be 100 %; e.g. PRVI=50 %.

Table 3. Amino acid substitutions between and character positions in A/Grebe/Novosibirsk/29/05, A/duck/Novosibirsk/56/05 and the group of A/Qinghai/05 strains of HPAI / H5N1.

Gene	Amino acid position	A/Grebe/ Novosibirsk/29/05	A/duck/ Novosibirsk/56/05	The group of A/Qinghai/05 strains
HA1	no substitutions			
Cleavage site *		**PQGERRRKKRGLF**	**PQGERRRKKRGLF**	**PQGERRRKKRGLF**
HA2	25	R	H	H
NA	29	I	M	M
	49-68 **	**deletion**	**deletion**	**deletion**
	143	V	L	V
NP	100	R	R	K
	403	A	T	A
NS1	**92** ***	**Q**	**Q**	**Q**
	201	I	I	F
NS2	no substitutions			
M1	no substitutions			
M2	no substitutions			
	31 ****	**S**	**S**	**S**
PB1	200	I	V	V
	296	H	Q	Q
PB2	69	E	A	E
	193	F	L	L
	221	T	A	A
	627 *****	**K**	**K**	**K**
	718	R	K	K
PA	618	A	T	T

* trypsin necessity for HA precursor cleavage and high level of pathogenecity (3, 14);
** belonging to subtype Z having increased tropism for poultry (5, 19);
*** resistance to antiviral action of interferons, tumor necrosis α-factor and increased virulence for pigs (17);
**** remantadine / amantadine sensitivity (6, 18);
***** increased virulence in mammal cells (15, 16).

Figure legends

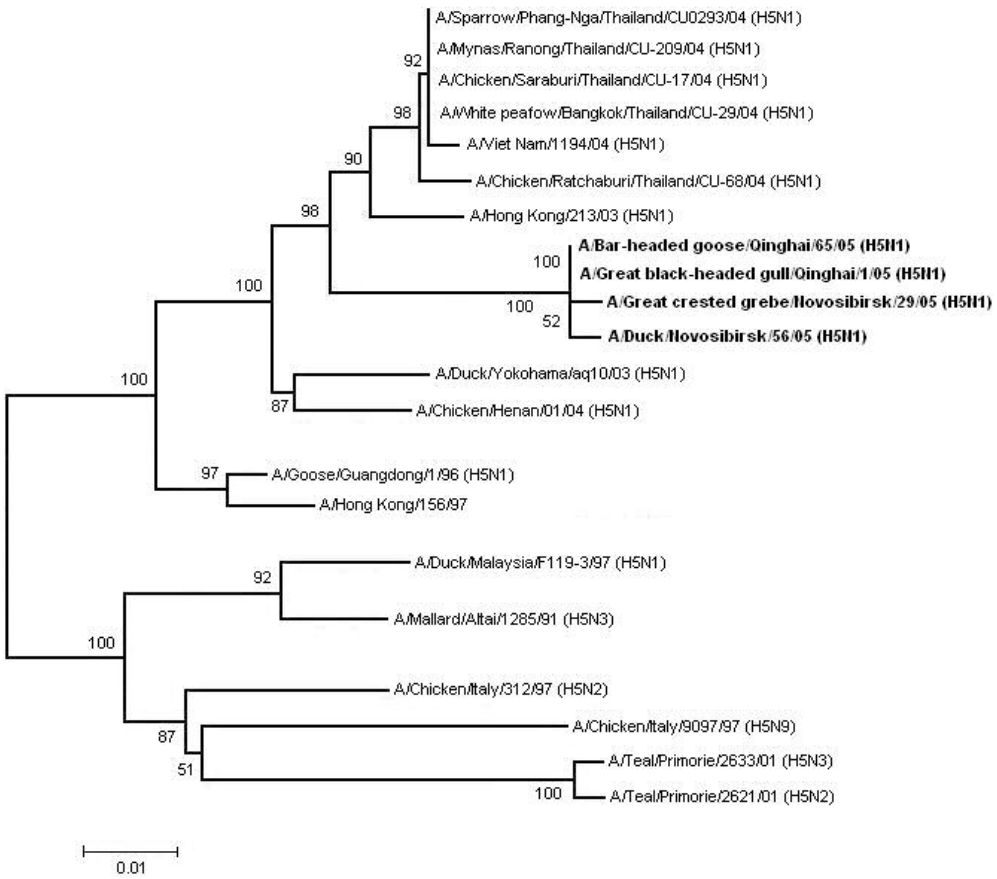
Figure 1. Genetic relationship between representative strains for HA gene.

Dear colleagues !

We are glad to present You our article concerning biological and genetic properties of Western Siberian HPAI / H5N1 strains isolated during 2005 summer epizootics. This article is a continuation of our previous article directed to EID.

Director of D.I. Ivanovsky Institute of Virology of Russian Academy of Medical Sciences

D.K. Lvov



Genetic relationship between representative strains for HA gene

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Biographical sketch of the first author

Dmitry Konstantinovich Lvov was born in June 26, 1931 in Moscow. He was graduated from Sanct-Petersburg Military Medical Academy in 1955. During 1955-1957 D.K. Lvov was working as junior researcher in the Military Institute of Sanitary (Moscow); 1957-1960 – in the Institute of parasitology and tropical medicine (Moscow); 1960-1967 – in the Institute of poliomyelitis and viral encephalitis (Moscow) as junior researcher, senior researcher and chief of laboratory. From 1967 till now D.K. Lvov is working in D.I. Ivanovsky Institute of Virology of Russian Academy of medical sciences; in 1987 D.K. Lvov became Director of this Institute.

D.K. Lvov is professor, full member of Russian Academy of medical sciences, editor in chief of "Voprosy Virusologii" journal, author of 11 books and more than 700 articles, scientific supervisor of 54 Ph.D., founder and real leader of ecology of viruses in Soviet Union and modern Russia.