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Isolation of high pathogenic avian influenza (HPAI) strains (H5N1) using embryo pig kidney cells (PS) and MDCK continuous cell lines from poultry and grebe (Podiceps cristatus) during epizootic outbreak in Western Siberia (July 2005)

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Isolation of highly pathogenic avian influenza (HPAI) strains (H5N1) using pig embryo kidney cells (PS) and MDCK continuous cell lines from poultry and grebe (*Podiceps cristatus*) during an epizootic outbreak in Western Siberia (July 2005)

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Abstract

Field materials were collected on July 28, 2005 from an avian disease outbreak with high mortality in the Novosibirsk region. After reverse transcriptase polymerase chain reaction (RT-PCR) analysis, influenza A/H5N1 RNA was detected in 100% of samples from dead and sick poultry, in 93% of samples from clinically healthy poultry in contact with sick birds, and in 35.2% of samples from clinically health wild birds in the same habitat.

Six strains of influenza A/H5N1 virus were isolated from wild birds and poultry in pig embryo kidney cells (PS) and MDCK cell lines, and were deposited in Russian State Collection of viruses (08.08.2005). The strains were identified using hemagglutination inhibition test (HIT), neutralization test (NT), RT-PCR and biochip-based approaches. Sequencing confirmed similarity between strains from wild birds and poultry, their belonging to high pathogenic avian influenza (HPAI) and neighboring to HPAI strains isolated 2005 spring in Qinghai Lake (China).

Epizootic outbreak among poultry occurred in the middle of 2005 July on the territory of Novosibirsk region (south of Western Siberia). It was necessary to establish etiological agent of this epizooty, isolate prototype strains, investigate their biological properties and deposit strains into Russian State collection of viruses. Presented article contains the results the referred program realization.

Methods

Collection of field materials was performed 28.07.2005 in a village of Zdvinsk Department of Novosibirsk region, near the center of epizootic outbreak. Samples from dead (cloaca and trachea swabs as well as brain, spleen, liver), sick (cloaca and trachea swabs as well as brain, spleen, liver, lungs, serum) and clinically healthy (cloaca and trachea swabs) poultry in contact with sick birds, as well as wild birds in the same habitat (Table 1) were placed into 2 ml tubes (Nunc, Denmark), which were transported and stored in liquid nitrogen in Duar vessels (ABS, USA).

Reverse transcriptase polymerase chain reaction (RT-PCR) for detection of influenza A virus RNA was conducted according to the standard technique using NP gene-specific primers (1).

Isolation of virus strains was performed using RT-PCR positive field samples (both cloaca/trachea swabs and tissue samples in parallel) in embryo pig kidney cells (PS) and MDCK continuous cell lines. Virus-induced cytopathological effect (CPE) was evaluated visually. After 24 and 72 h supernatant was taken for virus identification and subtyping.

Identification of influenza A virus strains was carried out by RT-PCR, hemagglutination inhibition test (HIT), neutralization test (NT) and biochip-based approaches.

Subtyping of influenza A viruses in field materials and isolated strains was performed by RT-PCR with subtype-specific primers and HIT with subtype-specific anti-sera

to determine hemagglutinin (HA) subtype as well as biochip-based approach to determine both HA and neuraminidase (NA) subtypes.

Sequencing of hemagglutinin gene (314 nucleotides (n.t.)) was performed using the sequencing apparatus ABI Prism 3130 (Applied Biosystems, USA) according to manufacturer's recommendations.

Comparative analysis of nucleotide sequences was carried out using software package «Lasergene» (DNASTAR Inc., USA).

Results and discussion

Utilizing RT-PCR, the presence of influenza A virus NP-gene RNA was observed in 100% of the samples from dead and sick poultry, and in 92.9% – from clinically healthy poultry in contact with dead and sick one. Among wild birds there was a total of 35.2% of samples found positive: mallards (*Anas platyrhynchos*) – 50.0%, pochards (*Aythya ferina*) – 36.4%, great crested grebes (*Podiceps cristatus*) – 50.0%, whereas coots (*Fulica atra*) and common terns (*Sterna hirundo*) were negative (Table 1). The prevalence of infected individuals among poultry (95.5%) compared to wild birds (35.2%) could be explained by the enhanced resistance of the latter against influenza as well as by the observation of local hunters that the poultry epizootic was followed by the mass deaths of wild birds.

In the positive samples, determination of the HA subtype was performed by RT-PCR and HIT, whereas the neuraminidase subtype by biochip-based approach. All positive samples were found to belong to H5N1 subtype of influenza A virus.

Six strains of influenza A/H5N1 virus were isolated using continuous PS and MDCK continuous cell lines from six birds – sick and dead domestic ducks, chickens and clinically healthy great crested grebe (Table 2). All strains were typed as H5N1. Strains were deposited into Russian State Collection of viruses functioning at the D.I. Ivanovsky Institute of

Virology: A/Grebe/Novosibirsk/29/05 (H5N1), A/Duck/Novosibirsk/56/05 (H5N1), A/Chicken/Novosibirsk/64/05 (H5N1), A/Chicken/Novosibirsk/65/05 (H5N1), A/Chicken/Novosibirsk/66/05 (H5N1), and A/Duck/Novosibirsk/67/05 (H5N1) (registration numbers 2372, 2371, 2373, 2374, 2375, and 2376, respectively) with the priority date August 08, 2005.

PS cell culture was turned to be very sensitive for reproduction of influenza A virus strain isolated from domestic birds, and in less extent was sensitive for reproduction of strain isolated from wild bird (Tables 2,3).

Sequencing revealed that hemagglutinin cleavage site of all isolated strains (Table 2) has a common sequence PQGERRRKKRGLF, with the multibasic amino acid stretch that is usual for highly pathogenic avian influenza (HPAI) strains (2,3). HA gene nucleotide sequences (314 n.t.) of all strains from poultry (both dead and sick) were identical (Figure 1) and their difference from A/Grebe/Novosibirsk/29/05 (H5N1) strains was only 2 nucleotide substitutions (from which one was silent). The extent of genetic identity of the isolated strains justifies the proposed link between viruses circulating among populations of wild birds and poultry. However, these strains differ from influenza A/H5 virus strains known before 2005 (Figure 1) illustrating the continuous evolution of H5 avian influenza.

Thus, it is most likely that the epizootic outbreak in Western Siberia (summer 2005) was provoked by H5N1 HPAI with the poultry infections caused by a virus circulating among wild aquatic birds.

Monitoring of influenza A virus circulation in wild biocenoses in the territory of Russia and neighboring countries had been conducted from 1971 until now (4-7). Virus strains belonging to 14 of the 16 known HA subtypes were isolated. The H5N2 subtype was previously found in the north-eastern part of Altai Krai (1991), H5N2 and H5N3 – in the south of Primorsky Krai (2001). Sequencing and cleavage site analysis showed that they belonged to low pathogenic avian influenza (LPAI) strains. The hypothesis was formulated

that the LPAI variants have been precursors for the H5 HPAI epizootic in Hong-Kong (1997) with the prognosis of the epizootic outbreak in the south-eastern Asia (2003-2005) (6,7).

Results of current study demonstrate once again the truth of hypothesis that there are wild aquatic birds, which are the natural reservoir of influenza A viruses, and poultry infections are the result of their contacts with wild birds (4,6-9). In the current case, H5N1 HPAI variants were introduced to the territory of Western Siberia from the endemic regions of south-eastern Asia and north-western China through "Dzhungarian Gate" during spring migration of birds with the further expansion of the epizootic after the increase in bird populations by juveniles and following of virus penetration into poultry. Phylogenetic data confirm high proximity of Qinghai (10) and Novosibirsk strains (Figure 1). The HPAI influenza variants could be introduced to the North Eurasia, to the places of mass nesting of aquatic and semi-aquatic birds. New waves of epizootic will probably occur during further season migrations of wild birds.

Distribution of HPAI variants in the extensive expanse of North Eurasia – the largest natural habitat of wild birds – could lead to an influenza A/H5N1 virus panzootic and to increased reassortment, which could result in the appearance of the pandemic influenza A variant able to circulate in human population.

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Table 1. Results of influenza A/H5N1 virus indication by RT-PCR of cloaca/trachea samples from birds collected in the epicenter of epizooty in Novosibirsk region (July, 2005).

Type	Clinical	Species of birds		Number of	Positive	
					number	%
Wild birds	without clinical features	Ducks	Mallard (*Anas platyrhynchos*)	12	6	50.0
	without clinical features		Pochard (*Aythya ferina*)	33	12	36.4
						40.0
	without clinical features	Great crested grebe (*Podiceps cristatus*)		2	1	50.0
	without clinical features	Coot (*Fulica atra*)		5	0	0
	without clinical features	Common tern (*Sterna hirundo*)		2	0	0
	TOTAL:	**5 species**		**54**	**19**	**35.2**
Domestic birds	without clinical features	Duck		14	13	92.9
				2	2	100.0
	sick			2	2	100.0
	died					
	died	Chicken		4	4	100.0
	TOTAL:	**2 species**		**22**	**21**	**95.5**
TOTAL:		**7 species**		**76**	**40**	**52.6**

Table 2. Results of influenza A virus strain isolation from birds collected near the center of epizootic in Novosibirsk region (July, 2005).

Strain	Source of isolation	Type of material for isolation		Cell line			Deposition into Russian State Collection of viruses	Accession number of GenBank database
				PS		MDCK		
		Cloaca / trachea swabs	Internals	HA-type according RT-PCR	NA-type according biochips	HA-type in HIT		
A/Grebe/ Novosibirsk/29/05	Great crested grebe without clinical features	—	+	H5	N1	H5	Registration number 2372 (priority date: 08.08.2005)	DQ190857
A/Duck/ Novosibirsk/56/05	Sick domestic duck	+	+	H5	N1	H5	Registration number 2371 (priority date: 08.08.2005)	DQ190858
A/Chicken/ Novosibirsk/64/05	Died chicken	+	+	H5	N1	H5	Registration number 2373 (priority date: 08.08.2005)	DQ190859
A/Chicken/ Novosibirsk/65/05	Died chicken	—	+	H5	N1	H5	Registration number 2374 (priority date: 08.08.2005)	DQ190860
A/Chicken/ Novosibirsk/66/05	Died chicken	+	+	H5	N1	H5	Registration number 2375 (priority date: 08.08.2005)	DQ190861
A/Duck /Novosibirsk/67/05	Died domestic duck	+	—	H5	N1	H5	Registration number 2376 (priority date: 08.08.2005)	DQ190862

Table 3. Infectious titers of influenza A/H5N1 virus strains (3-rd passage) for PS continuous cell line.

Strain	Source of isolation	lg $TCID_{50}$/0.2 ml	RT-PCR
A/Grebe/Novosibirsk/29/05	Great crested grebe without clinical features	5.0 [1]	+
A/Duck/Novosibirsk/56/05	Sick domestic duck	7.0 [2]	+
A/Chicken/Novosibirsk/64/05	Died chicken	10.5 [2]	+
A/Chicken/Novosibirsk/65/05	Died chicken	10.0 [2]	+
A/Chicken/Novosibirsk/66/05	Died chicken	10.0 [2]	+

[1] With trypsin in culture media for infected cells.

[2] Without trypsin in culture media for infected cells.

Figure legends

Figure 1. Phylogenetic tree hemagglutinin gene nucleotide sequences (314 n.t.) of influenza A/H5 strains.

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.

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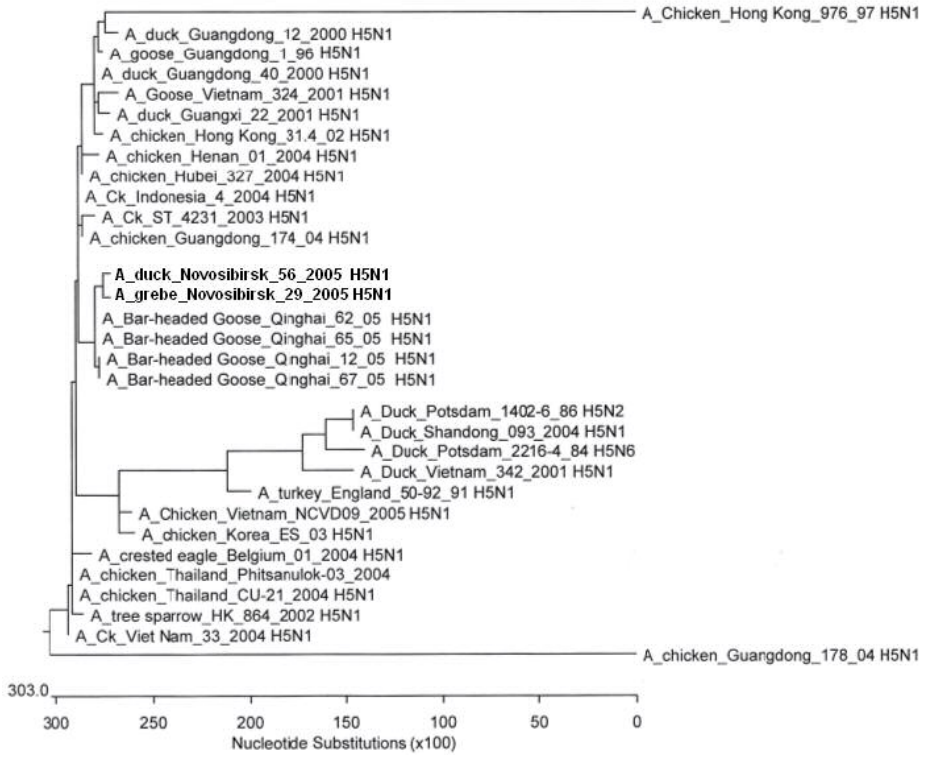

Figure 1. Phylogenetic tree hemagglutinin gene nucleotide sequences (314 n.p.) of influenza A/H5 strains.
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