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The use of microarrays for the identification of the origin of genes of avian influenza viruses in wild birds

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ABSTRACT

Forty-two strains of avian influenza viruses were isolated from the wild waterfowls' feces in the city of Moscow. These viruses, as well as reference strains and some experimental reassortants, were analyzed by microarrays. The microarrays contained 176 probes to the different segments of influenza virus genome. The microarray helps to determine 1) the hemagglutinin and neuraminidase proteins subtype; 2) the primary structure of the C-terminal sequence of the viral NS1 protein, which serves as a ligand for the PDZ domain; 3) the presence of stop codons in the reading frame of PB1-F2 as well as the N66S substitution in the PB1-F2 viral protein; 4) the presence of the polybasic site for hemagglutinin cleavage. The viruses of the H3N1, H3N6, H3N8, H4N6, H1N1, H5N3, and H11N9 subtypes were identified from the group of wild birds' isolates. All isolates contained the ESEV sequence at the C-terminus of the NS1 protein and the full-length reading frame for the PB1-F2 protein. The replacement of N66S in PB1-F2 was found in six strains. However, the presence of the ESEV sequence (ligand of PDZ domain) in the NS1 virus protein and the N66S substitution in PB1-F2 did not lead to the pathogenicity of these viruses for mice. All isolates demonstrated high yield growth in chicken embryos and were infectious and immunogenic for mice, but did not induce any clinical symptoms.

INTRODUCTION

Wild aquatic birds represent the major natural reservoir of influenza viruses, which includes the viruses with 16 currently known hemagglutinin (HA) antigenic subtypes and 9 neuraminidase (NA) subtypes. These viruses are highly adapted to their hosts and usually cause no sign of the disease. They replicate in the intestine and are efficiently transmitted by the fecal-oral route through contaminated water [1]. Although the virus is actively replicated in the host and intensively secreted by the host into the environment, the host does not suffer from it due to the long adaptation of the virus to the host. This is beneficial to the virus, since active birds effectively spread the infection. High pathogenicity usually occurs in unnatural artificial ecosystems with a high density of objects of infection. Examples of such ecosystems are poultry farms, where highly pathogenic avian influenza viruses (HPAIV) occur. Analysis of the evolution of influenza viruses shows that HPAIV are usually located at the tips of young evolutionary branches of viruses, because the viruses that kill the hosts, are ceased to exist themselves [2].

Low pathogenic avian influenza viruses (LPAIV) of wild birds are located in the bases of the evolutionary branches of influenza A viruses. They evolve slowly and retain a number of characteristic features. These features include: the conservative structure of the HA receptor-binding site, the structure of the cleavage site, which is digested by the trypsin-like proteases, but not by the intracellular proteases; low pH of activation of the HA, associated with its high resistance to the acidic environment of the digestive tract of birds.

High pathogenicity is a multifactor characteristic, which is determined by multiple changes in many genes. Those changes include the following: appearance of a polybasic sequence in the HA cleavage site of HPAIV [3]; deletion in the stem section of NA [4]; the E627K mutation in the polymerase protein PB2 that increases the rate of virus replication [5-7]; substitution of N66S in PB1-F2 protein that accelerates the nuclear transport [8-10]; and the substitutions in the nonstructural protein NS1 of HPAIV that lead to the efficient suppression of the host interferon synthesis [7, 11-13].

A *Biogripp* microarray [14] that contains 176 probes to different segments of the genome of influenza viruses was developed at the Engelhardt Institute of molecular biology (Moscow, Russia). This microarray can be used to

monitor and characterize influenza viruses. The microarray allows:

1) to identify the RNA of the influenza virus in biological materials and to determine the type and subtype of the viruses;
2) to determine the virus resistance to anti-influenza drugs – amantadine and rimantadine – as well as to the neuraminidase inhibitors (oseltamivir and its analogs);
3) to determine the presence and the structure of the PDZ-domain ligand in the NS1 protein: ESEV, EPEV, ESKV, and KSEV sequences that are typical for the HPAIV, or RSKV and RSEV sequences that are characteristic for the low pathogenic strains;
4) to determine the presence of a polybasic cleavage site of HA, which allows the virus to multiply in the internal organs of the host, and is typical for the H5 and H7 HPAIV;
5) to determine the presence of stop codons at positions 12 and 58 in *PB1-F2* gene as well as mutation N66S in the corresponding protein.

We describe here the results of analysis of the forty-two strains of avian influenza viruses that were isolated from the feces of wild waterfowl in the city of Moscow. These viruses as well as reference strains and some experimental reassortants were analyzed by means of the *Biogripp* microarray in order to determine the origin of their genes.

MATERIALS AND METHODS

Viruses

Viruses were isolated from the avian feces collected on the shore of a pond in the city of Moscow in the period from 2006 to 2014. They are stored in the virus repository of the Chumakov scientific center (Moscow, Russia).

The Vietnam/1203/04-PR8/CDC-RG (H5N1) (VN-PR) virus was generated by reverse genetics in the Influenza branch of the Centers for Disease Control and Prevention (CDC, USA). It contains *HA* and *NA* genes from the A/Vietnam/1203/2004 (H5N1) virus and the rest of the genes from the A/Puerto Rico/8/34 (PR8) virus. The segment of the *HA* gene encoding the polybasic cleavage site of this virus was modified. This virus was kindly provided by Dr. R. Donis. The A/Hamburg/5/2009 virus was kindly provided by Dr. M. N. Matrosovich (Institute of Virology, Philipps University, Marburg, Germany). The cold-adapted A/Leningrad/134/17/57 (H2N2) virus was kindly provided by Prof. L. G. Rudenko (Institute of Experimental Medicine, St. Petersburg, Russia). The A/mallard/Sweden/91/2002 (H7N9) virus was kindly provided by Dr. R. A. Fouchier (Erasmus Medical Centre, Rotterdam, The Netherlands). The A/duck/Buryatia/664/1988 virus was obtained from the virus repository of the Gamaleya Scientific Research Institute of Epidemiology and Microbiology (Moscow, Russia). The full names of the viruses and their designations are shown in Table 1.

Isolation and propagation of influenza viruses

Samples of avian feces were resuspended in two volumes of phosphate buffered saline (PBS) containing 0.4 mg/ml gentamycin, 0.1 mg/ml kanamycin, 0.01 mg/ml amphotericin B, and 2% MycoKill AB (PAA Laboratories GmbH) and centrifuged at 4000 rpm for 10 min. The obtained supernatants were used to inoculate 10-day old embryonated chicken eggs (CE) (0.2 ml per each). After incubation for 48 h, the allantoic fluids were collected and tested by hemagglutination assay with chicken erythrocytes. The samples that gave a positive reaction in a hemagglutination assay were selected for the consecutive propagation. Three serial passages were performed for the isolation of viruses. Viruses were propagated in CE at 36°C. Virus-containing allantoic fluids were harvested 48 h post infection. Cold-adapted strains were propagated at 32°C and harvested 90 h post infection. The virus infectivity was determined by titration in the CE and expressed as $\log_{10}$ EID_{50}.

Viral RNA sequencing

Viral RNA was extracted from allantoic fluid using QIAamp Viral Mini Kit (Qiagen, Germany). cDNA fragments generated by reverse transcription followed by PCR were sequenced using 3130x/Genetic Analyzer (Applied Biosystems, USA) with BigDye Terminator V3.1 Sequencing Kit. GenBank accession numbers for the sequences are listed in Table 1.

Microarray test

The *Biogripp* microarray is a plate with an array of plastic hybridization microchambers (volume of 30 µl) containing covalently immobilized oligonucleotide probes. cDNA was synthesized from viral RNA by reverse transcription with MMLV enzyme (Promega, USA) using the primer uni12 (5'-AGCAAAAGCAGG-3'). The fluorescently labeled PCR products were placed in the chamber. In the course of hybridization, the labeled fragments of the influenza virus genome formed a complex with the immobilized complementary probe. The hybridization results were analyzed using the universal hardware-software complex TU 9443-004-02699501-2006. The fluorescence signal in each well was recorded.

Pathogenicity for mice

Six weak-old BALB/c mice were used in this experiment. Groups of 10 mice were anesthetized and then inoculated intranasally with virus or placebo. The virus doses that were tested ranged from 2.0 to 6.0 $\log_{10}$ EID_{50}/mouse. Mouse survival and their body weight in both experimental and control groups of animals were monitored daily. On day 15 post-infection, serum samples were collected for antibody titration. The levels of antibody in sera were determined by ELISA with anti-mouse IgG horseradish peroxidase labeled second antibody (Sigma-Aldrich, USA). All of the studies with highly pathogenic A/Chicken/Kurgan/3/05 virus were conducted in a BSL-3 containment facility.

Table 1. Influenza A viruses used in this study.

#	Virus	Designation[a]	Subtype	GenBank accession number (Gene)	PB1-F2 66[b]	*NS1* line[c]
1	A/duck/Buryatia/664/1988	d/664	H3N1	MF969261, MF969262 (*HA*, *NA*)	N	A
2	A/duck/Primorie/3628/2002	d/3628	H9N2	DQ787797, DQ787799, DQ787800 (*HA*, *M*, *NS*)	N	A
3	A/mallard/Sweden/91/2002	m/Sw	H7N9		N	A
4	A/gull/ Moscow/3100/2006	g/3100	H6N2	EU152234–EU152241	N	A
5	A/duck/Moscow/3554/2008	d/3554	H3N1	GU991376, MF969260 (*HA*, *NA*)	N	A
6	A/duck/Moscow/3556/2008	d/3556	H3N1		N	A
7	A/duck/Moscow/3661/2008	d/3661	H4N6	MF680290–MF680297	N	A
8	A/duck/Moscow/3641/2008	d/3641	H11N9	GU991377 (*HA*)	N	A
9	A/duck/Moscow/3806/2009	d/3806	H3N8	CY120775 (*HA*)	N	B
10	A/duck/Moscow/3735/2009	d/3735	H4N6	CY120772, MF422091–MF422097	N	B
11	A/duck/Moscow/3740/2009	d/3740	H4N6	CY120773, MF422098–MF422104	N	B
12	A/duck/Moscow/3799/2009	d/3799	H4N6	CY120774, MF422105–MF422111	N	B
13	A/duck/Moscow/3720/2009	d/3720	H6N2	CY120771 (*HA*)	N	B
14	A/duck/Moscow/4242/2010	d/4242	H3N8		S	A
15	A/duck/Moscow/4298/2010	d/4298	H3N8		N	A
16	A/duck/Moscow/4203/2010	d/4203	H3Nx		N	A
17	A/duck/Moscow/4238/2010	d/4238	H3N6		N	A
18	A/duck/Moscow/4182/2010	d/4182	H5N3	KF885672–KF885679	N	A
19	A/duck/Moscow/4206/2010	d/4206	H5N3		N	A
20	A/duck/Moscow/4031/2010	d/4031	H6N2		S	A
21	A/duck/Moscow/4494/2011	d/4494	H3N8		S	A
22	A/duck/Moscow/4521/2011	d/4521	H3N8		S	A
23	A/duck/Moscow/4522/2011	d/4522	H3N8		S	A
24	A/duck/Moscow/4681/2011	d/4681	H3N8		S	A
25	A/duck/Moscow/4661/2011	d/4661	H3N8		N	A
26	A/duck/Moscow/4518/2011	d/4518	H4N6	MF673524–MF673531	N	A
27	A/duck/Moscow/4528/2011	d/4528	H4N6	MF673532–MF673539	N	A
28	A/duck/Moscow/4641/2011	d/4641	H4N6	MF422112–MF422119	N	A
29	A/duck/Moscow/4652/2011	d/4652	H4N6	KX518711–KX518718	S	A
30	A/duck/Moscow/4643/2011	d/4643	H4N6	KX509943–KX509950	N	A
31	A/duck/Moscow/4524/2011	d/4524	H3N2		N	A
32	A/duck/Moscow/4771/2012	d/4771	H4N6	MF673540–MF673547	N	A
33	A/duck/Moscow/4772/2012	d/4772	H4N6		N	A
34	A/duck/Moscow/4781/2012	d/4781	H4N6	KX530510–KX530517	N	A
35	A/duck/Moscow/4843/2012	d/4843	H4N6	MF673548–MF673555	N	A
36	A/duck/Moscow/4844/2012	d/4844	H4N6		N	A
37	A/duck/Moscow/4788/2012	d/4788	H3N8		N	A
38	A/duck/Moscow/4780/2012	d/4780	H3N8		N	B
39	A/duck/Moscow/4952/2013	d/4952	H5N3		N	A
40	A/duck/Moscow/4971/2013	d/4971	H5N3		N	A
41	A/duck/Moscow/4970/2013	d/4970	H1N1		N	B
42	A/duck/Moscow/5037/2014	d/5037	H3N8		N	A
43	A/Leningrad/134/17/57	Len	H2N2		N	A
44	A/Puerto Rico/8/34	PR8	H1N1		N	A
45	A/Hamburg/5/2009	Hamb	H1N1		N	A
46	Vietnam/1203/04-PR8/CDC-RG	VN-PR	H5N1		N	A
47	A/chicken/Kurgan/3654at/2005	ch/Ku	H5N1	HQ724520–HQ724527	N	A
	Experimental reassortants					
48	VN-PR × Len (cl.3697)	3697	H5N2		N	A
49	VN-PR × Len (cl.4760)	4760	H5N2		N	A
50	Hamb × Len (cl.4885)	4885	H1N1		N	A
51	Hamb × Len (cl. 4886)	4886	H1N1		N	A
52	Hamb × Len (cl.4888)	4888	H1N1		N	A

[a] Brief designation of the virus.

[b] Amino acid at position 66 in the PB1-F2 protein.

[c] The *NS1* gene line according to [15].

Construction of reassortants

In order to obtain cold-adapted reassortants, 10-day old CE were simultaneously infected with the donor of attenuation A/Leningrad/134/17/57 (Len) and a donor of surface proteins in a dose of 7.0 $\log_{10}$ EID_{50} each. The CE were incubated for 18 h at 32°C, followed by another passage for 18 h at 32°C. The aliquots of the harvested virus were incubated with mouse serum against Len virus overnight and used for the consecutive passage. The samples that were positive in the hemagglutination inhibition test with serum against the surface protein donor and negative with serum against Len were harvested. The virus was subsequently passaged three times for 96 h at 26°C using the limiting dilutions method.

RESULTS

Investigated viruses

Samples from gull and mallard feces were collected on the shore of the pond in Troparevo Park in Moscow. Forty-two strains of the avian influenza viruses were isolated from nearly 2000 samples that were collected in the fall of 2006-2014 (Table 1). All of these viruses demonstrated high yield growth in CE and were not pathogenic for mice [16]. Three viruses were tested in chickens and demonstrated a lack of pathogenicity [17]. The genomes of several viruses were partially or fully sequenced [18]. All viruses were analyzed on the *Biogripp* microarray.

The following viruses were used as the reference strains in this study: A/duck/Buryatia/664/1988 (H3N1), A/duck/Primorie/3628/2002 (H9N2), A/mallard/Sweden/91/2002 (H7N9), attenuated in the laboratory A/chicken/Kurgan/3654at/2005 (H5N1) strain [19] as well as H1N1, H2N2, and H1N1pdm human viruses and 5 experimental reassortants.

The main characteristics and designations of the investigated viruses are shown in Table 1.

The HA and NA subtypes of all the viruses were determined by means of microarray analysis.

The genes encoding the HA and NA proteins of several viruses were sequenced. For all these viruses, the results of subtyping on the microarray matched the sequencing data.

Pathogenicity factors of natural isolates

The polybasic HA cleavage site was identified in a single case – for the H5 HA of the ch/Ku virus, which corresponds to the sequencing data.

The resistance to amantadine and its analogues caused by substitutions in the M2 protein was found only in human influenza viruses: PR8 (A27 and N31) and Hamb (N31).

In all isolates from wild birds, the NS1 protein is terminated by the ESEV sequence, while in the strains ch/Ku, PR8 and Len - by the ESKV, RSEV, and RSKV, respectively, that is confirmed by the sequencing data (GenBank).

The stop codon in the reading frame of the PB1-F2 protein was found in the Hamb virus, which corresponds to the sequencing data.

The replacement N66S in the PB1-F2 protein, which is considered as a factor increasing the pathogenicity of the virus, was identified in eight strains (Table 1). For the 15 viruses included in this study, the *PB1* genes were sequenced and the results of analysis using the microarray matched the sequencing data.

In order to determine how the replacement N66S in the PB1-F2 protein affects the pathogenicity of natural isolates, we performed an animal study with viruses of

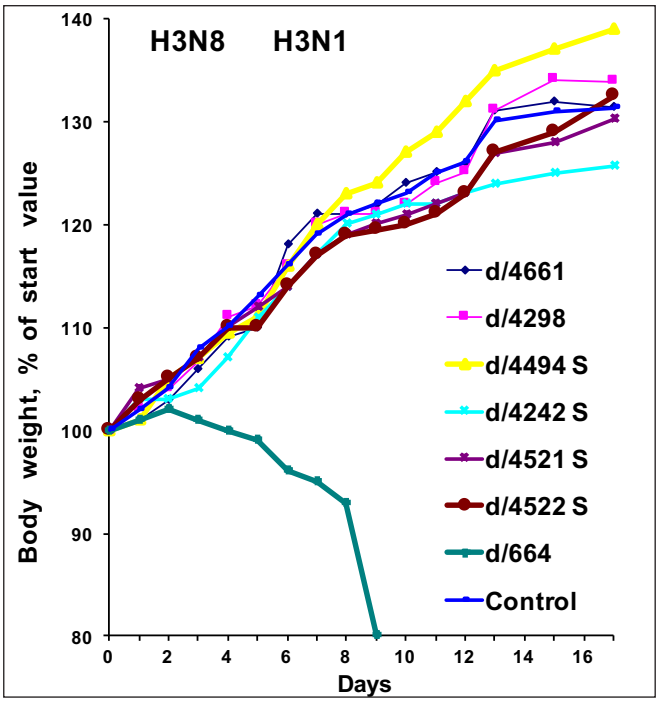

Fig. 1. The dynamics of weight change of mice infected with the viruses d/4661, d/4298, d/4494, d/4242, d/4521, d/4522, and d/664. Viruses with N66S substitution in PB1-F2 are labeled with the letter S.

Table 2. Pathogenicity and immunogenicity for the mice of avian influenza viruses that differ in amino acids at position 66 of the PB1-F2 protein

Virus	Subtype	PB1-F2 66[a]	LD_{50}[b]	GMT[c]
d/4242	H3N8	S	>6.0	2523
d/4494	H3N8	S	>6.0	3215
d/4521	H3N8	S	>6.0	3594
d/4522	H3N8	S	>6.0	2163
d/3554	H3N1	N	>6.0	1983
d/664	H3N1	N	1.8	10000
d/4298	H3N8	N	>6.0	1193
d/4661	H3N8	N	>6.0	3519
d/4182	H5N3	N	>6.0	1748
d/4206	H5N3	N	>6.0	4213
d/4641	H4N6	N	>6.0	420
d/4652	H4N6	S	>6.0	515
g/3100	H6N2	N	>6.0	312
d /3720	H6N2	N	>6.0	783
d/4031	H6N2	S	>6.0	406

[a] Amino acid at position 66 in the PB1-F2 protein.

[b] LD_{50} – the dose, measured in $\log_{10}$ EID_{50}, resulting in the death of 50% of the mice (average for three experiments).

[c] The geometric mean antibody titer (GMT) in ELISA.

different subtypes that have mutations at this position. The data for the influenza viruses of H3N8 and H3N1 subtypes are shown in Fig. 1. Similar results were obtained for the viruses of the H4N6 and H6N2 subtypes (Table 2). The dynamics of weight change of mice infected with duck viruses having N66 and S66 in the PB1-F2 protein did not differ significantly from that of the mice in the control group. It is important to emphasize that all infected mice showed a powerful virus-specific immune response, indicating that the infection was effective, but did not lead to the development of clinical symptoms. The only duck virus that caused the disease and death of mice was the virus isolated in 1988 d/664 (H3N1).

Determination of the genome composition of experimental reassortants

Since the *Biogripp* microarray contains a large number of probes to each of the genes of influenza viruses, the resulting hybridization patterns of evolutionarily distant viruses produced on this microarray are usually very different. For example, the hybridization patterns of PR8, Len, and Hamb viruses with 14 probes for the *NS* gene significantly differ from each other, and from the avian influenza viruses (Fig. 2). The binding patterns with 15 probes for *M* gene and 14 probes for *PB1* gene are also noticeably different (Fig. 3 and 4). These results enabled us to use the *Biogripp* microarray for the determination of the genome composition of the experimental reassortants of influenza viruses.

A classical way to produce attenuated strains for live influenza vaccine is the reassortment of the epidemic virus with the cold adapted donor of attenuation. Reassortment is achieved by the simultaneous infection of CE with two parental strains and subsequent cultivation under selective conditions [20]. Sometimes it is not easy to obtain a reassortant of the desired composition with two genes from an epidemic virus and six genes from the donor of attenuation. The control of the genome composition of obtained reassortants is an important practical task.

To solve this problem, the Express-Mix-PCR method was developed in the Institute of Experimental Medicine (St. Petersburg, Russia), which enables the determination of the composition of the reassortant genome [21]. The determination of the genome composition using the microarray has a number of significant advantages: it does not require specific primers for all the genes of analyzed viruses, generates results quickly, and has a low assay cost.

In this study, we analyzed the *HA*, *NA*, *PB1*, *NS*, and *M* genes of five experimental reassortants. The viruses VN-PR (H5N1) and Hamb (H1N1) were used as the surface protein donors. The attenuation donor was the cold-adapted Len (H2N2) virus. The microarray binding assay enabled us to explicitly determine the origin of the *HA*, *NA*, *PB1*, *NS*, and *M* genes and to identify the mixtures that contained genes from both parents. As an example of such an analysis, the binding patterns of the parental (Len and Hamb) and reassortant (4886) viruses with probes to the gene encoding the NS1 protein are shown in Fig. 5. These results show that the pattern of the *NS1* gene of the clone 4866 is an overlay of the corresponding gene patterns of Len and Hamb viruses, which means that the clone 4866 contains a mixture of *NS* genes from both parents.

All the experimental reassortants contained the gene encoding the HA protein from the surface proteins donor. H5 reassortants contained the *NA* N2 gene from the Len donor, while the reassortants of the Hamb virus contained the *NA* N1 gene from the parent Hamb strain. Only two clones (3697 and 4888) contained the genes *PB1*, *NS*, and *M* from the Len donor of attenuation. The clone 4760 contained the *PB1* gene from the PR8 virus and a mixture of *M* genes from Len and PR8 viruses. The

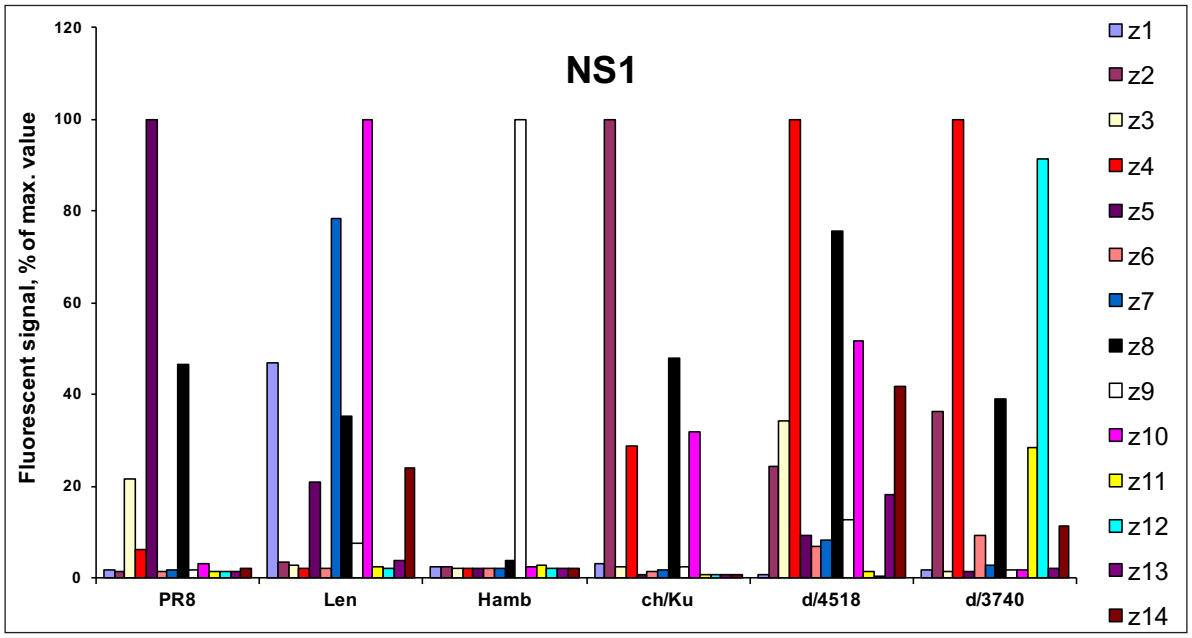

Fig. 2. Six hybridization patterns of viruses with 14 probes to the gene that encodes the protein NS1.

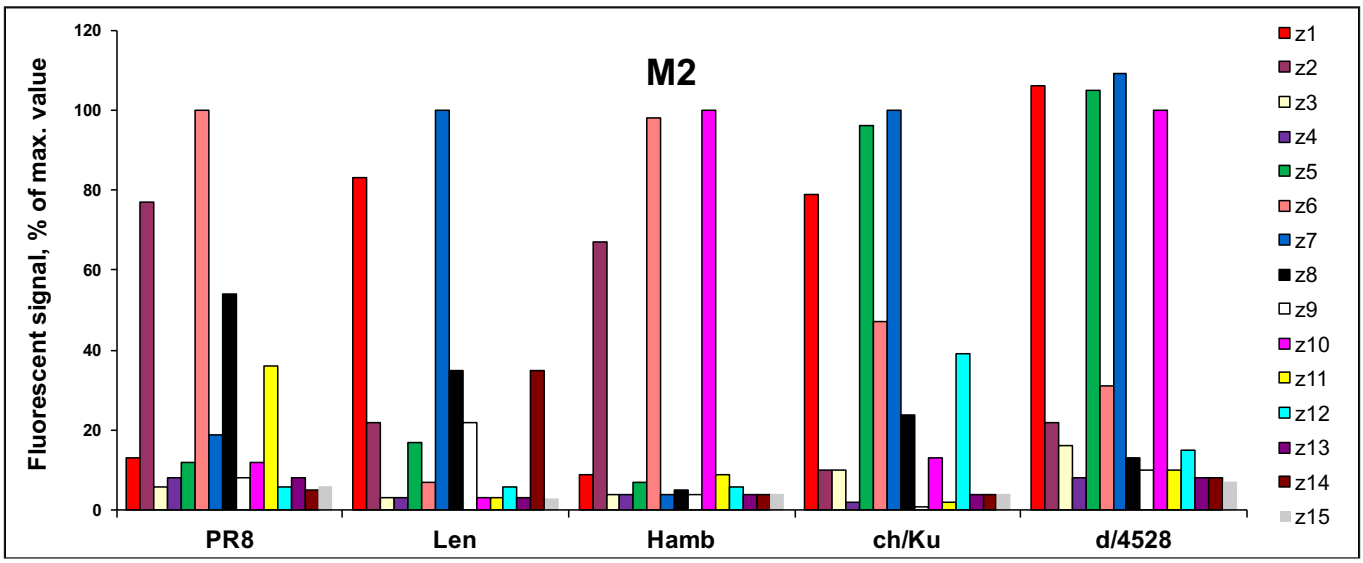

Fig. 3. Five hybridization patterns of viruses with 15 probes to the gene that encodes the M2 protein.

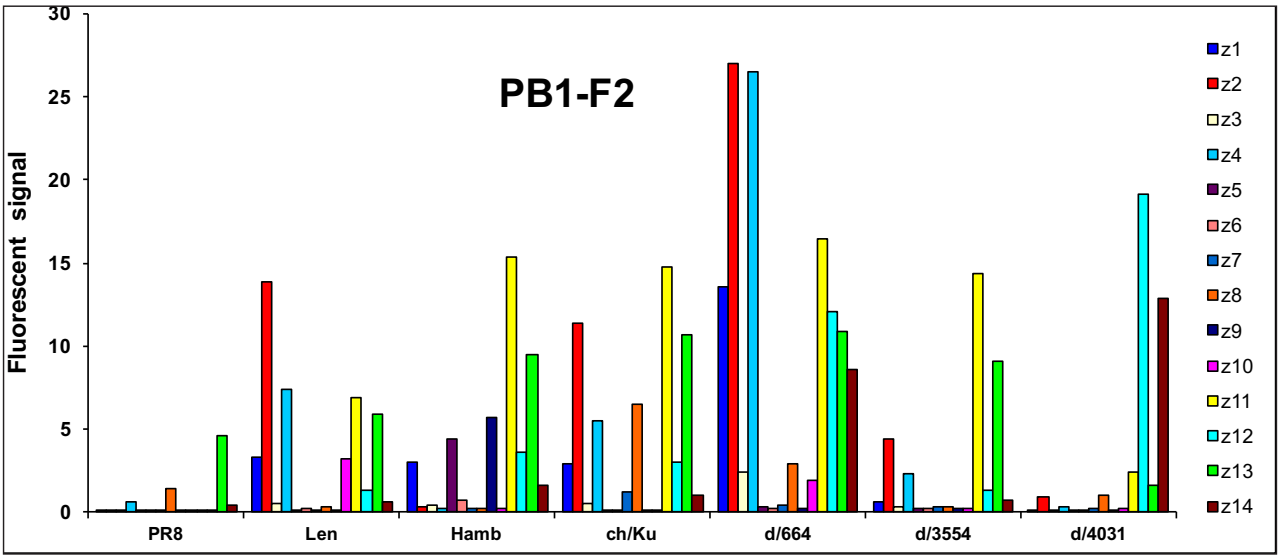

Fig. 4. Seven hybridization patterns of viruses with 14 probes to the gene that encodes the protein PB1-F2.

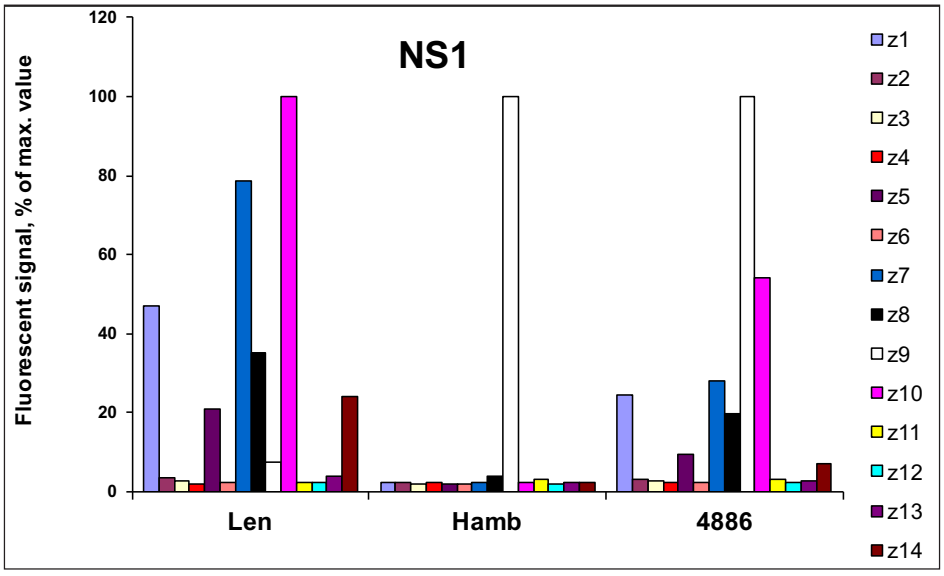

Fig. 5. Hybridization patterns of the parental (Len and Hamb) viruses and the reassortant virus (clone 4886) with probes to the gene that encodes the NS1 protein.

clone 4885 contained *PB1* and *M* genes from the Hamb virus and the mixture of *NS* genes from both parents. The clone 4886 contained *PB1* gene from the Len virus, and the *NS* and *M* genes from both parents (Table 3).

Determination of the origin of genes of avian influenza viruses in wild birds

The results obtained in the previous section suggest that the *Biogripp* microarray can be used to determine the evolutionary origin of genes in the natural isolates of influenza viruses. The analysis showed that, according to the hybridization patterns of avian viruses with probes z7-z14 for the *NS1* gene, the viruses are clearly divided into two groups. Most isolates practically do not interact with probes z11 and z12, and effectively bind to probes z8, z9, z10, and z14. The viruses of the other group, on the contrary, effectively bind to the probe z12 and do not interact with probes z9 and z10. The hybridization patterns of the first and second groups are presented by the isolates d/4518 and d/3740, respectively (Fig. 2).

The *NS* gene sequencing revealed that viruses from different groups contain the *NS1* gene of two different evolutionary lines A or B [15]. These results explain the different hybridization patterns of viruses that belong to different groups. The evolutionary lines A and B have divided about 200 years ago. They differ by eight nucleotides in the 655-700 region of *NS1* gene that is recognized by probes z8-z12 of the microarray. Thereby the analysis of viruses using the microarray enabled us to determine the evolutionary line of *NS1* genes for all of the viruses.

The binding patterns of H5N1 and H5N3 influenza viruses with probes to H5 *HA* gene were significantly different for the low pathogenic and high pathogenic strains: low pathogenic wild duck viruses effectively bind to probes z3 and z4, whereas highly pathogenic strains ch/Ku and VN-PR (H5N1) predominantly interact with probe z1 (Fig. 6). Thus, the analysis of the binding pattern with probes to H5 *HA* as well as the determination of the virus cleavage site structure can be used for the identification of highly pathogenic viruses.

The virus d/664 was of special interest since it is the only influenza virus in our practice that was isolated from wild ducks and was found to be pathogenic for mice. This virus showed a significant difference in the hybridization pattern with the probes to H3 *HA* compared to all of the other H3 viruses. The binding pattern with probes to the N1 *NA* gene was similar to a pattern of highly pathogenic H5N1 viruses, and the PB1-F2 pattern resembled a pattern of human H2N2 influenza virus (Figs. 4, 7, 8). Of course, by using this information we are not able to reveal the causes of the pathogenicity of the d/664 virus at this time, but the obtained data are sufficient enough to pay special attention to this virus. In the future, we plan to carry out the sequencing of the complete genome of this virus.

DISCUSSION

In this study, we examined 42 natural isolates of the influenza A virus of the H1N1, H3N1, H3N2, H3N6, H3N8, H4N6, H5N3, H6N2, H7N9, H9N2, and H11N9 subtypes for the presence of the known to date pathogenicity factors that are described in the literature [9, 10, 12]. Our results showed that markers like the ESEV sequence of the PDZ domain ligand in the NS1 viral protein and changes in the reading frame of the viral protein PB1-F2 (N66S replacement) in the context of the wild duck influenza virus genome are not associated with the virus pathogenicity for mice. All of the viruses in this study successfully infected mice and caused a strong immune response, but did not lead to the disease – no clinical symptoms were recorded. This once again confirms that pathogenicity is a complex and multifactorial property. From all of the studied viruses, only ch/Ku (H5N1), VN-PR (H5N1), and d/664 (H3N1) viruses caused the death of mice at a low dose of infection. The first of them, ch/Ku (H5N1), is the classical HPAIV, despite the presence of N66 in the PB1-F2 protein. Its pathogenicity is determined by the polybasic sequence in the HA cleavage site which is the sufficient but not the necessary condition

Table 3. Characteristic features of parental and reassortant viruses.

Virus	HA	NA	PB1-F2		NS1			M2	
			Stop codon[a]	Pattern[b]	PDZ ligand	Stop codon[a]	Pattern[b]	A.a. 27, A.a. 31[c]	Pattern[b]
Len	H2		–	Len	RSKV	+7	Len	V, S	Len
PR8	H1	N1	–	PR8	RSEV	0	PR8	A, N	PR8
Hamb	H1	N1	11, 57	Hamb	–	-11	Hamb	V, N	Hamb
VN-PR	H5	N1	–	PR8	RSEV	0	PR8	A, N	PR8
Reassortants									
3697	H5	N2	–	Len	RSKV	+7	Len	V, S	Len
4760	H5	N2	–	PR8	RSKV	+7	Len	A/V, N/S	PR+Len
4885	H1	N1	11, 57	Hamb	RSKV	-11, +7	Len+Hamb	V, N	Hamb
4886	H1	N1	–	Len	RSKV	-11, +7	Len+Hamb	V, N/S	Len+Hamb
4888	H1	N1	–	Len	RSKV	+7	Len	V, S	Len

[a] The numbers indicate the positions of the stop codons in the gene. (–) Absence of a stop codon in the reading frame of PB1-F2. 0 is the standard position of the stop codon in the reading frame NS1.

[b] Characteristic hybridization patterns of viruses with probes are designated as: Len, PR8, and Hamb.

[c] Amino acids at position 27 and 31 of M2 protein.

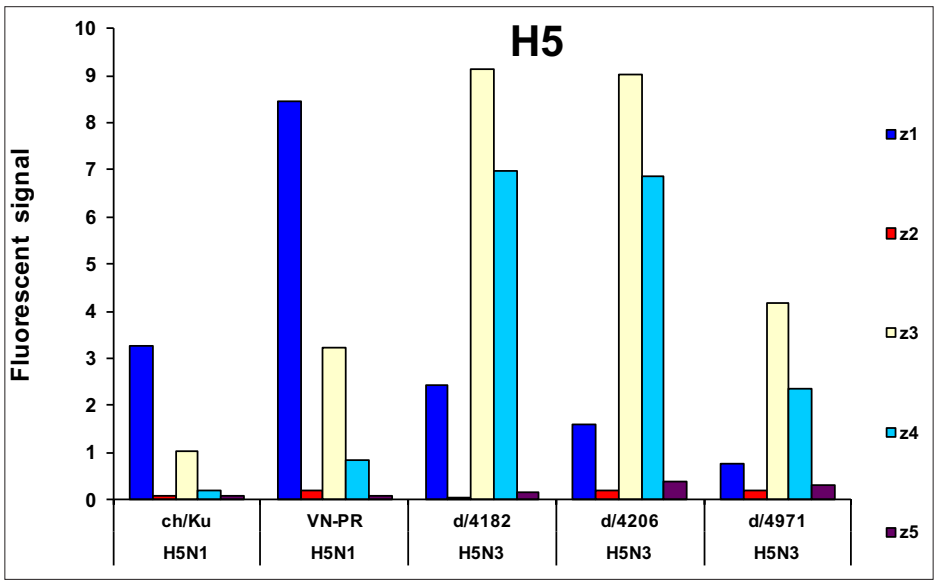

Fig. 6. The hybridization patterns of viruses with probes for the *HA* H5 gene.

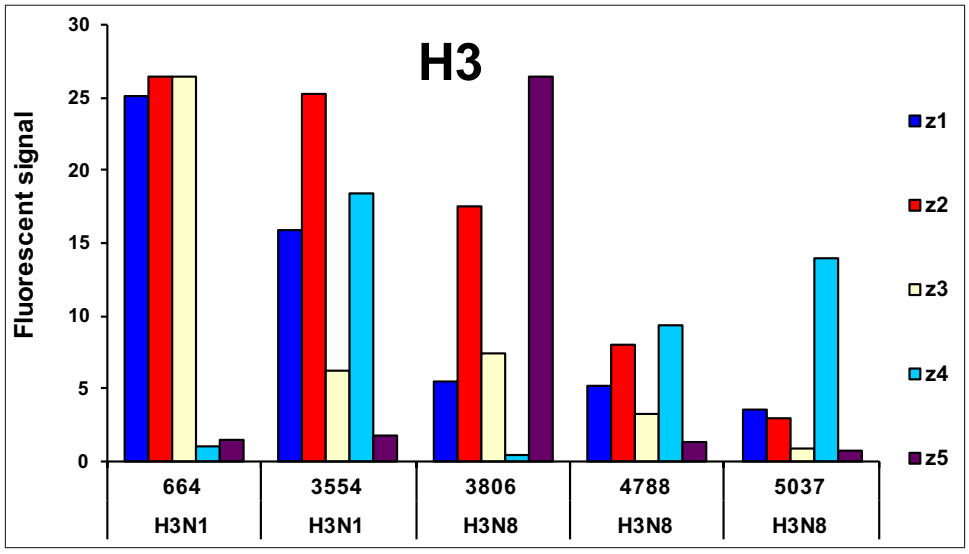

Fig. 7. The hybridization patterns of viruses with probes for the *HA* H3 gene.

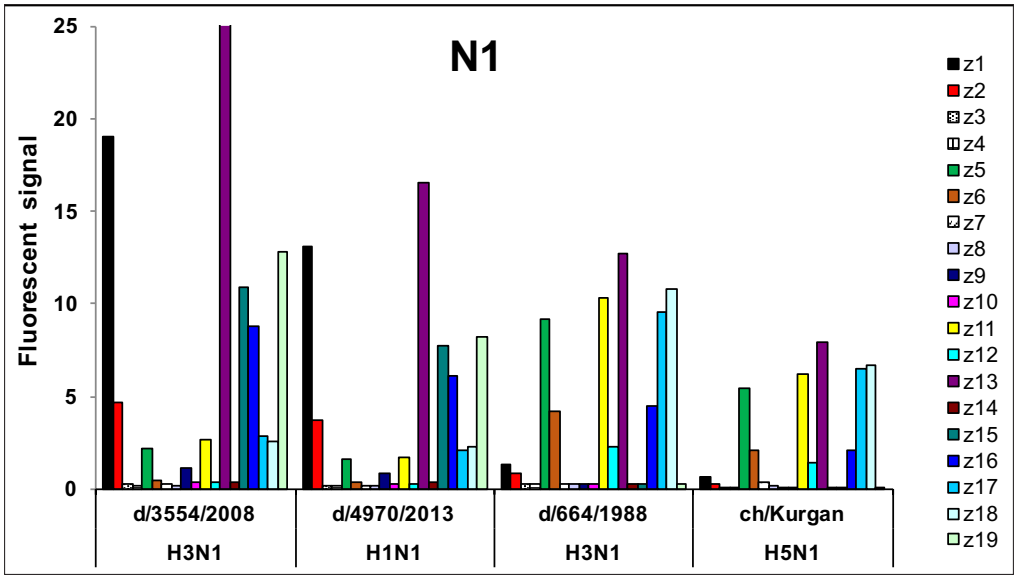

Fig. 8. The hybridization patterns of viruses with probes for the *NA* N1 gene.

for the pathogenicity of the virus. The VN-PR does not have a polybasic site and the other formal markers of pathogenicity – it contains NS1 protein, terminated with RSEV, N66 in PB1-F2 protein, and HA is cleaved only by secretory serine proteases. However, the pathogenicity of this virus for mice only insignificantly differs from that of ch/Ku, which causes the death of mice at a dose of 2.0 $\log_{10}$ EID_{50}. The last highly pathogenic virus from our collection – d/664 – does not differ by the above-mentioned markers from other H3N1 viruses, which do not cause any clinical symptoms in mice. Human virus A/Hamburg/5/2009 caused the disease of mice with weight loss despite the presence of the following attenuating factors: the absence of the PB1-F2 protein and the absence of the PDZ domain ligand due to the shortening of the NS1 protein (unpublished data).

Thus, the pathogenicity is not defined by individual amino acid substitutions, but is rather a polygenic trait. The *Biogripp* microarray can be used for the determination of HA and NA subtypes. In some cases, analysis using this microarray enables distinguishing the evolutionary lines of genes that code for the surface and internal proteins of influenza viruses. The *Biogripp* microarray can be used for monitoring the evolutionary branches of different genes and allows for recognizing the potentially dangerous influenza viruses in circulation.

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CONFLICT OF INTEREST

The authors do not pursue commercial or financial interests.

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