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Original Article

Oligonucleotide microchip for subtyping of influenza A virus

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Background Influenza A viruses are classified into subtypes depending on the antigenic properties of their two outer glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Sixteen subtypes of HA and nine of NA are known. Lately, the circulation of some subtypes (H7N7, H5N1) has been closely watched because of the epidemiological threat they present.

Objectives This study assesses the potential of using gel-based microchip technology for fast and sensitive molecular subtyping of the influenza A virus.

Methods The method employs a microchip of 3D gel-based elements containing immobilized probes. Segments of the HA and NA genes are amplified using multiplex RT-PCR and then hybridized with the microchip.

Results The developed microchip was validated using a panel of 21 known reference strains of influenza virus. Selected strains represented different HA and NA subtypes derived from avian, swine and human hosts. The whole procedure takes 10 hours and enables one to identify 15 subtypes of HA and two subtypes of NA. Forty-one clinical samples isolated during the poultry fall in Novosibirsk (Russia, 2005) were successfully identified using the proposed technique. The sensitivity and specificity of the method were 76% and 100%, respectively, compared with the 'gold standard' techniques (virus isolation with following characterization by immunoassay).

Conclusions We conclude that the method of subtyping using gel-based microchips is a promising approach for fast detection and identification of influenza A, which may greatly improve its monitoring.

Keywords Hemagglutinin, H5N1 subtype, influenza A virus, microarray analysis, neuraminidase.

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Introduction

Influenza viruses pose a major challenge for public health and continue to be a leading cause of respiratory tract infection, resulting in significant morbidity, mortality, and financial burden.

Influenza viruses belong to the family Orthomyxoviridae. They have an envelope, and their genome consists of eight fragments of linear single-stranded antisense RNA. Based on the differences in their nucleoproteins and membrane proteins, they are divided into three major types, A, B, and C. Among these, type A is the most dangerous from the epidemiological point of view.[1]

Influenza viruses A are further classified according to antigenic properties of two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). So far 16 subtypes of HA (H1–H16) and nine subtypes of NA (N1–N9) have been described.[2] Viruses belonging to all subtypes are found in wild populations of aquatic birds, which are therefore their natural reservoir. In contrast to the birds, only three subtypes of HA (H1–H3) and two subtypes of NA (N1 and N2) usually circulate in human populations. However, lately, infections of humans by other subtypes, including H7N7, H9N2 and H5N1, have become more frequent.

In 1997, the first cases of human infections with subtype H5N1 were found during an outbreak of influenza among birds in Hong Kong. These cases were highly lethal: out of 18 infected patients, six died.[3] In 2004 new cases of infection with the H5N1 (the so-called 'bird flu') occurred in Asian countries, resulting in 32 deaths in Vietnam and Thailand[4] and forcing the extermination of millions of domestic fowl.[5] In 2005 migrating wild birds spread the highly pathogenic H5N1 to northern China, Mongolia, Tibet, Kazakhstan, and Russia. Currently the bird flu is spreading further to European countries, the Middle East, and the Caucasian region. From the beginning of 2004 to April 2007, a total of 287 people were infected with H5N1, of whom 168 died according to a WHO report.[4] The potential ability of the bird flu type A H5N1 to cause a pandemic is uncertain, and this unpredictable nature of the current situation calls for the development of sensitive, reliable, and fast methods of identification of virus subtypes. These methods will play a crucial role in the whole complex of prophylactic measures.

Within the last decade a wide range of molecular methods have been proposed for fast identification of the subtypes of influenza virus. Most of them employ the amplification of viral nucleic acids by polymerase chain reaction (PCR) and differ by their approach to the detection of its products: it may be real-time PCR, immuno-PCR, multiplex PCR, isothermic PCR, or a combination thereof.[6–12] The main difficulties in the development of these methods are the design of large multiplex sets of primers and the analysis of PCR products by traditional methods. These factors limit the number of subtypes that can be identified in a single experiment.

The technology of oligonucleotide microchips makes it possible to run a multi-parametric analysis of genetic material and is therefore a promising approach to simultaneous identification of all possible subtypes of the influenza virus A. However, many of the existing techniques based on the use of oligonucleotide microchips identify just a few of existing subtypes of HA and NA.[13–16] A more advanced microchip developed recently by Lodes *et al.*[17] covers almost all subtypes and is highly reliable. However, this technique is quite laborious, and the manufacturing of high density microchips and their scanning require sophisticated and costly equipment. For these reasons, it could not be easily adapted to clinical laboratories and field applications.

Here we propose another method of typing of influenza virus A based on oligonucleotide microchip (biochip) with 3D gel elements. The method consists of amplification of the viral genes of HA and NA and subsequent hybridization of the amplified fragments on the biochip. We describe an optimized set of diagnostic oligonucleotides and report the results obtained from test samples, as well as primary samples obtained from sick birds.

Materials and methods

Viral samples

Reference viral strains were obtained from the Collection of Viruses of the D. I. Ivanovsky Institute of Virology. Stock viruses were diluted 10-fold and injected into the allantoic cavity of 10-day-old embryonated chicken eggs. Four eggs were inoculated per dilution. After 48 hours at 37°C post-inoculation, the chorioallantoic fluid was collected and tested for hemagglutination with 0.5% chicken red blood cells. The virus titer was determined by the Reed–Muench method.[18] Strains were used when their titer was at least 10^6 units per ml of 50% egg infective dose (EID_{50}). Strains used in this work are listed in Table 1.

For analytical sensitivity studies human viral isolates A/WSN/33(H1N1) and A/Victoria/3/75(H3N2) with known EID_{50}/ml were serially diluted in PBS. At each concentration, three samples were prepared. RNA was isolated for each sample and then subjected to amplification and hybridization stages. The analytical sensitivity was determined as the lowest EID_{50} at which all three samples from both isolates were successfully detected.

Field samples (cloacal swabs and mixture of internal organs pieces) were collected in PBS/glycerol transport medium following WHO guidelines (WHO/CDS/CSR/NCS/2002.5 Rev. 1) and were kept frozen at −70°C until shipping. Upon arrival the samples were investigated with multiple methods, including viral culture, sequencing, and microchip analysis.

Nucleic acid isolation and PCR amplification

For RNA isolation, 200 μl of viral isolate (in PBS) or 200 μl of field sample (in transport medium) were treated with either a commercial kit (Narvac, Moscow, Russia), or

Table 1. Strains of the influenza virus type A used in the study

Strain	Serotype	Accession number
A/WSN/33	H1N1	J02176
A/USSR/90/77	H1N1	X00027
A/Puerto Rico/8/34	H1N1	ISDN13422
A/Brazil/11/78	H1N1	X86657
A/New Caledonia/20/99	H1N1	AY289929
A/Beijing/262/95	H1N1	AY289928
A/Pintail Duck/Primorie/695/76	H2N3	AF290442
A/Laughing gull/New Jersey/75/85	H2N9	AF116201
A/duck/Ukraine/1/63	H3N8	V01087
A/Victoria/3/75	H3N2	V01086
A/England/42/72	H3N2	AF201875
A/Udorn/307/72	H3N2	M54895
A/Sydney/5/97	H3N2	AJ311466
A/Duck/Czechoslovakia/56	H4N6	AF290436
A/Mallard Duck/Pensylvania/10218/84	H5N2	AF100180
A/Duck/Ho Chi Minh/14/78	H5N3	AF290443
A/FPV/Rostock/34	H7N1	M24457
A/FPV/Weybridge/34	H7N7	L37794
A/Swine/Hong Kong/9/98	H9N2	AY428485
A/Chicken/Germany/49	H10N7	M21647
A/Pilot whale/Maine/328/84	H13N2	M26091

TRI® Reagent (Sigma, Saint Louis, MO, USA) according to the manufacturer's instructions.

The samples were hybridized in two stages. In the first stage, cDNA was synthesized, and segments of HA and NA genes were amplified using two pairs of primers: r173ha 5′-AGA AAC AAG GGT GTT TT-3′ and f817ha 5′-GA ATG ATH GAY GGN TGG TAT G-3′ (H = A, C or T; Y = C or T); and R556na 5′-AGT AGA AAC AAG GAG TTT TTT-3′ and F552na 5′-TGT GTT TGC AGA GAT AAT TGG-3′. Twenty five microliters of the reaction mix contained 2.4 mm $MgCl_2$, 80 mm KCl, 16 mm Tris–HCl, pH 9.0, 0.2 mm of each dNTP, 5 U of Taq DNA polymerase, 25 U of M-MLV reverse transcriptase (both from Sileks, Moscow, Russia), 20 U of RNasin (Promega, Madison, WI, USA), 100 nm of each of the primers f817ha, r556na, f552na, 200 nm of the primer r173ha, and 5 μl of viral RNA sample. Amplification was carried out in thermocycler 'Tercyc' (DNA-Technology, Moscow, Russia). The reaction mixture was incubated at 50°C for 30 minutes, denatured at 94°C for 3 minutes, and 31 cycles of 20 seconds at 94°C, 30 seconds at 58°C, and 45 seconds at 72°C were performed. At the end, the mixture was incubated at 72°C for 5 minutes. Amplification products were analyzed by gel electrophoresis in 2% agarose. They contained the two expected products: 640 bp long segment of the HA gene and 600 bp long segment of the NA gene. One microliter of the first reaction mix was used for the second round of amplification.

In the second stage, the following primers were used: R173ha* and F170ha (5′-TTTGAATTCTACCACAAGTGTGA-3′) for HA gene and R556na* and F551na (5′-ATGGTGTTTGGATGGGAAGAAC-3′) for NA gene. They produced amplified fragments 300 and 390 bp long, correspondingly. Reverse primers R173ha* and R556na* used at this stage carried fluorescent label at their 5′-end designated by an asterisk. To obtain mostly single-stranded fluorescently labeled fragments, the reverse primers were used at a 10-fold excess relative to direct primers. The reaction mixture contained in 25 μl 70 mm Tris–HCl, pH 8.6, 16.6 mm $(NH_4)_2SO_4$, 2.5 mm $MgCl_2$, 0.2 mm of each dNTP, 5 U of Taq DNA polymerase, 10 nm of primers f170ha and f551na and 100 nm of primers r173ha* and r556na*. After 3 minutes of denaturation at 94°C, the amplification was carried out for 35 cycles of 20 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C. At the end, the mixture was incubated at 72°C for 3 minutes.

Preparation of oligonucleotides and microchips

All oligonucleotides were synthesized on a 394 synthesizer (Applied Biosystems, Foster City, CA, USA). For subsequent gel immobilization, a free amino group was introduced using 3′-Amino-Modifier C7 CPG 500; for fluorescent labeling, 5′-Amino-Modifier C6 was used (Glen Research, Sterling, VA, USA). Fluorescent label IMD-504 (Biochip-IMB, Ltd., Moscow, Russia) was introduced according to the manufacturer's instructions. Oligonucleotides for immobilization and PCR were designed using programs 'Oligo 6' (Molecular Biology Insights, West Cascade, CO, USA) and 'Bioedit' (Ibis Therapeutics, Carlsbad, CA, USA).

Sequences of the immobilized diagnostic oligonucleotides are listed in Table 2. Biological microchips were manufactured as described earlier.[19]

Hybridization

Hybridization was carried out by adding 12 μl of the reaction mixture obtained after the second round of amplification to 24 μl of 1.5 m guanidine thiocyanate, 0.075 m HEPES pH 7.5, and 7.5 mm EDTA. The mixture was injected into hybridization chamber and incubated at 37°C for 5.5 hours. After hybridization, the biochip was washed with water at 37°C three times for 30 seconds each and air-dried.

The results of the hybridization were recorded using computer-assisted device 'Chip Detector' (Biochip-IMB, Ltd.). The results were processed using software package ImaGeWare supplied with the detecting equipment.

Results

We designed a microarray of oligonucleotides immobilized in gel elements – a biochip – for molecular typing of influenza. The call set of the oligonucleotides identifies 15 subtypes of HA and two subtypes of NA. The biochip consists of 47 gel elements, of which 41 contain test probes and six contain control probes. The scheme of the biochip is shown in Figure 1.

Selection of subtype-specific oligonucleotides was performed by multiple alignment of the sequences of HA and NA of various serotypes isolated from birds and mammals, including humans. These sequences were selected from GenBank. The number of oligonucleotides included in the call set for individual subtypes depends on the number and distribution of conserved sequences within the amplified segments and on the extent of variations (Figure 2). In some cases, reliable identification of a given subtype required the inclusion of all possible variations within the same conserved segment, i.e. several oligonucleotides with similar, but not identical sequences. For instance, subtype H1 is identified using three oligonucleotides, $H1_1$, $H1_2$, and $H1_3$ (Figure 2). $H1_1$ and $H1_2$ are complementary to two variants of the subtype H1 and are separated by 27 nucleotides. At the same time, $H1_2$ and $H1_3$ cover the same sequence and differ by two nucleotides only. In most cases, the sequences of oligonucleotides were chosen to prevent their cross-hybridization with several subtypes. The two exceptions from this rule are oligonucleotides H7/H10/H15 and H3/H4.

Table 2. Immobilized oligonucleotides used for Influenza A virus subtyping

Oligo-nucleotide*	Sequence 5′ → 3′	Sequence position	Strain accession number
H1.$_{1}$	CTC CCT GGG GGC AAT CAG	1632–1649	CY015580
H1.$_{2}$	GAT TTT GGC GAT CTA TTC AAC	1584–1604	CY015580
H1.$_{3}$	GAT TCT GGC GAT CTA CTC AAC	1584–1604	CY015580
H2.$_{1}$	AAA TTG AGC AGC ATG GGG GTT	1592–1612	CY015135
H2.$_{2}$	AAA TTG AGC AAY ATG GGG GTT	1592–1612	CY015135
H3.$_{1}$	TGY TTT TTG CTT TGT GTT GTT	1626–1645	CY016108
H3.$_{2}$	CTT ATG ACC ATG ATG TAT ACA	1510–1530	CY016108
H3.$_{3}$	GAA GCA TTA AAC AAC CGG TT	1536–1555	CY016108
H3.$_{4}$	ATT TCC TTT GCC ATA TCA TG	1608–1627	CY016108
H3/H4	ATT TCA TTC GCC ATA TCA TG	1608–1627	CY016108
H4	CGT WGC ACT RCT TTT AGC CTT	1623–1643	D90302
H5.$_{1}$	TCA ACA GTG GCG AGT TCC	1617–1634	AF046080
H5.$_{2}$	TCT ACG GTG GCG AGT TCC	1617–1634	AF046080
H5.$_{3}$	CAA TGG GAA CTT ACC AAA TAC	1585–1605	AF046080
H5.$_{4}$	CAA TGG GCA CTT ATC AGA TAC	1585–1605	AF046080
H5.$_{5}$	CAT GGT AGC TGG TCT RTC TTT	1649–1669	AF046080
H6	ATA GTA CGG TAT CGA GCA GT	1604–1623	CY015451
H7.$_{1}$	GAG GCA ATA CAA AAC AGA ATT CA	1543–1565	CY015014
H7.$_{2}$	CAA TGC ARA ATA GAA TAC AGA TTG A	1547–1571	CY015014
H7/H10/H15	TTT AGC TTC GGG GCA TCA TGT	1615–1635	CY015014
H8	GGC GGC CAG TCT TTG CTT	1608–1625	D90304
H9.$_{1}$	TGT CGC CTC ATC TCT TGT G	1590–1608	AB256706
H9.$_{2}$	TTC TGG GCC ATG TCC AAT G	1636–1654	AB256706
H10	GGC TCT TCT GAA TAG ACT GAA C	1534–1555	M21647
H11	AGA TTC TAG TGG GAA TGT G	1592–1610	CY014719
H12	GCA TCT ACA GCA GTG TTG CC	1608–1627	CY014598
H13	AAC GTT TAC AAA GCA TTR TC	1617–1636	M26091
H14	CTT TGT CTT CGT GGC ACT GAT T	1643–1664	CY014604
H15	AAT AGG ATA ATG ATC AAT C	1573–1591	CY006032
N1.$_{1}$	TTT GAR ATG ATT TGG GAT CC	1131–1150	DQ376693
N1.$_{2}$	TTT GAR ATG GTT TGG GAT CC	1131–1150	DQ376693
N1.$_{3}$	GGA TAC AGC GGG AGT TTT AT	1221–1240	DQ376693
N1.$_{4}$	GGG TAC AGC GGA AGT TTC GT	1221–1240	DQ376693
N1.$_{5}$	GGA TAT AGC GGG AGT TTT GT	1221–1240	DQ376693
N2.$_{1}$	TGY ATC AAY AGG TGT TTT TAT G	1253–1274	CY016118
N2.$_{2}$	TGY ATC AAT MGG TGC TTT TAT G	1253–1274	CY016118
N2.$_{3}$	TCT GGT ATT TTC TCT GTT GA	1223–1242	CY016118
N2.$_{4}$	GCA GAT AAA TAG GCA AGT CAT	1174–1194	CY016118
N2.$_{5}$	GCA GAT CAA TAG ACA AGT CAT	1174–1194	CY016118
N2.$_{6}$	GCA GAC CAG CAG ACA AGT CAT	1174–1194	CY016118
N2.$_{7}$	GCA GGT CAA TAG ACA GGT CAT	1174–1194	CY016118

*The designations of oligonucleotides correspond to their location in Figure 1.

Hybridization of the biochip with fluorescently labeled DNA obtained by reverse transcription of viral RNA and amplification of the product results in the accumulation of fluorescent signal in the gel element corresponding to the original subtype. Because of the sequence variability within individual subtypes, there may be a different number of positive signals within the group of elements corresponding to a single subtype. At the same time, gel elements corresponding to other subtypes accumulate imperfectly matched and therefore unstable complexes. These complexes emit much weaker fluorescent signals. Based on the accumulated statistical data, the signals were considered positive when their integral fluorescence exceeded by at least threefold the average signals of the three control gel elements (f4–f6). The threshold value was calculated as described earlier.[20] Signals that did not exceed the threshold level were considered negative. The sequences of the immobilized oligonucleotides and the conditions of hybridization were optimized to allow for reliable interpretation of the resulting pattern by computer processing of the image as

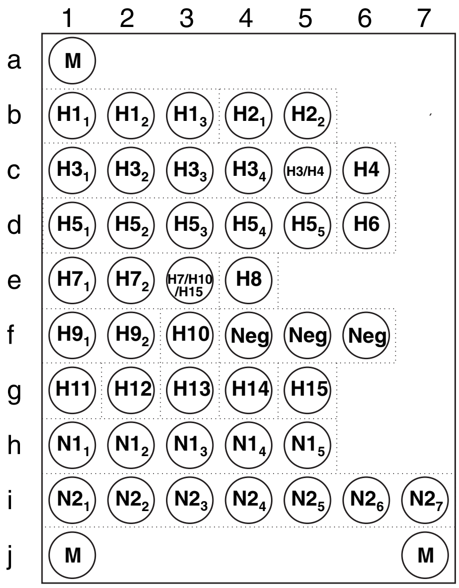

Figure 1. Scheme of the biochip for molecular typing of influenza A virus. Rows b through g contain oligonucleotides for typing of 15 subtypes of HA, while rows h and i identify subtypes 1 and 2 of NA. Immobilized oligonucleotides are designated as follows: letters H or N indicates HA or NA, correspondingly; next, there is a number corresponding to a subtype; the subscript number is an arbitrary designation of the diagnostic oligonucleotides when there is more than one for the identification of a single subtype. Corner gel elements M (a1, j1, and j7) contain immobilized IMD504 fluorescent dye and serve as marker for computer processing of hybridization pattern. Elements f4 through f6 represent empty gel pads and serve as controls of background fluorescence of the gel elements.

well as visual inspection. Namely, the positive signals within the certain group from the HA and NA parts of the microchip provide the hybridization pattern corresponding to the specific subtype.

The discrimination power of the diagnostic biochip was assessed using RNA samples from 21 reference strains described in Table 1. Three examples of this analysis are illustrated in Figure 3. For each individual HA probe, all positive fluorescent signals fall into one group only. As only two NA subtypes are represented on the chip, corresponding signals either fall into one of these group (panels A and C) or are all negative. In all cases the typing was in full concordance with the known subtypes of the viruses determined by traditional immunological methods.

The strains used for hybridizations illustrated in Figure 3 were isolated at different times and locations. Figure 3(A) shows fluorescent pattern after the hybridization of biochip with amplified fragments of HA and NA genes of the strain A/USSR/90/77(H1N1). Among HA elements, positive fluorescent signals are visible in elements b1 and b3. They are both members of the group corresponding to the H1 subtype of HA. Signals from the elements which are members of other groups of the H1 cluster do not exceed the threshold level and are close to the signals of negative control elements f4–f6. Within the NA cluster, positive signals are observed only within the group of elements located at positions h1–h5 and corresponding to the N1 subtype of the NA gene.

Figure 3(B) shows the hybridization with another sample – amplified fragments of HA and NA of the strain A/Duck/Czechoslovakia/56(H4N6). In the HA cluster, a positive fluorescent signal is observed only in the group of elements c5–c6, which identifies subtype 4 (H4). Within the NA cluster, all signals are close to the background level, indicating the absence of perfect matches between the sequences of the sample and immobilized probes. Therefore, the NA gene of the analyzed strain does not belong to either of the first two subtypes. Finally, Figure 3(C) shows the result of the hybridization with the amplified segments of the A/Swine/Hong Kong/9/98(H9N2) strain. Here positive signals register in groups of elements f1–f2 (corresponding to the H9 subtype of HA) and i1–i6 (subtype N2 of NA). Signals from other elements do not exceed the threshold level.

Importantly, the analysis of all 21 strains isolated from birds, animals, and humans never resulted in cross-hybridization of the amplified fragments with elements belonging to different groups within HA- or NA-clusters. In most cases, positive signals exceeded the intensity of negative control elements five- to 20-fold. This extent of quantitative difference allows for unambiguous visual identification of positive signals and therefore subtyping of the samples. This subtyping by visual inspection of the images was often further facilitated by the emergence of several positive signals within the same group of elements containing diagnostic oligonucleotides for the same subtype.

After testing the subtyping biochip using reference samples of viruses obtained by cultivation on chick embryos, we analyzed 41 RNA samples isolated from cloacal swabs and suspension of internal organs (brain, liver, spleen) of two sick domestic fowls, six which recently died and 33 'healthy' (the birds were in infected area but had not any clinical symptoms) during an outbreak in Novosibirsk region in July 2005. These field specimens were also characterized by isolation in the MDCK cell line with subsequent immunological subtyping and sequencing.[21] Comparison of the data obtained using microchips to the results of the conventional immunotyping (Table 3) revealed five false-negative samples. In all these five cases RNA was isolated from cloacal swabs of birds without clinical symptoms. All eight pooled samples of internal organs of sick and dead birds tested positive by both methods.

Figure 4 shows the pattern of hybridization on the biochip using DNA amplified from one of these samples. Within the HA-cluster of gel elements, positive fluorescent

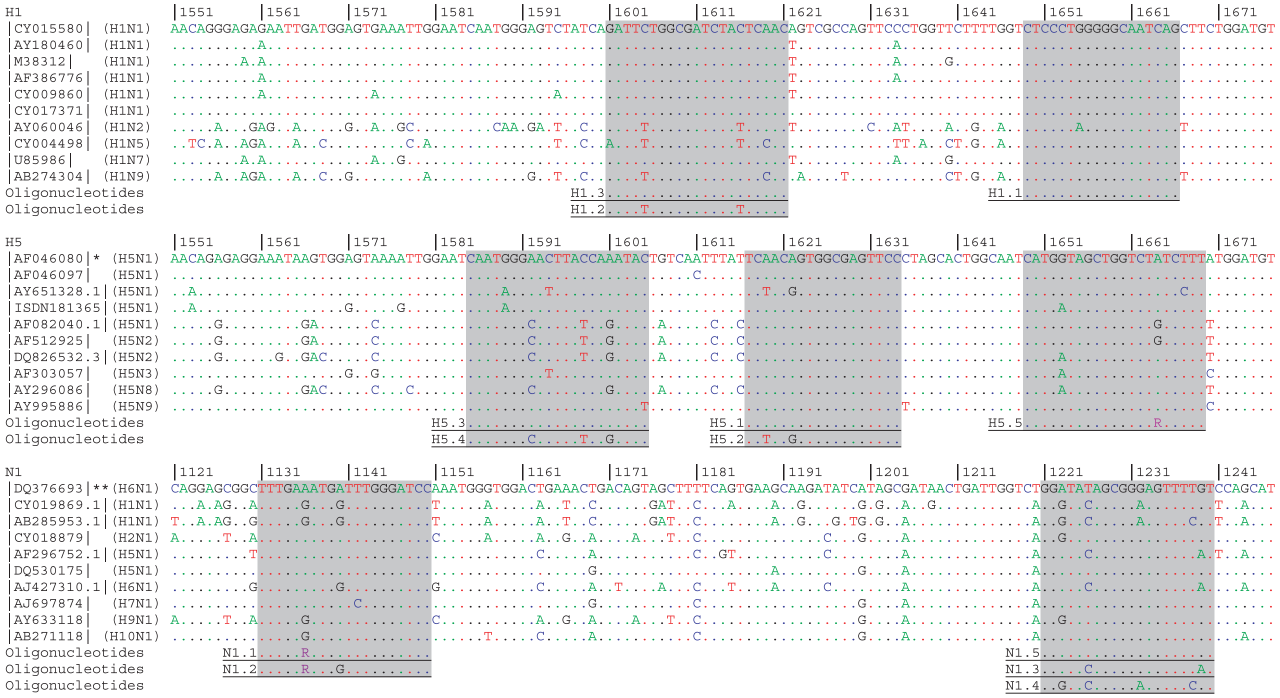

Figure 2. Nucleotide sequence alignment of HA and NA gene fragments of different influenza A subtypes (H1Nx, H5Nx, HxN1) with designed oligonucleotides indicated. Genomic regions covered by variant oligos are shown in gray. Nucleotide designation reads as follows: dot (.), same as reference sequence; R, A or G. Nucleotide positions are numbered according to reference sequences: (*) for H1, H5 subtypes and (**) for N1 subtype. The designations of oligonucleotide probes are at the left from them and correspond to their location in Figure 1.

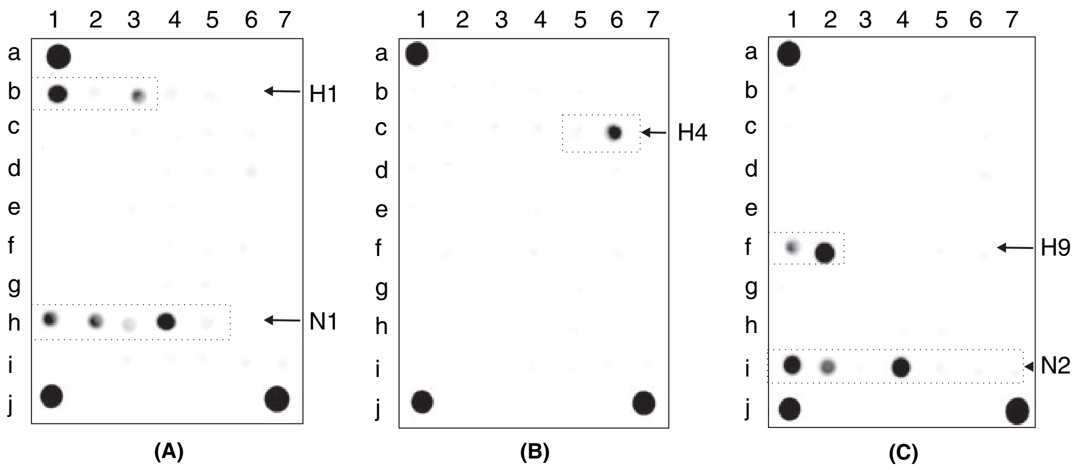

Figure 3. Hybridization patterns obtained using reference samples of influenza virus A/USSR/90/77(H1N1) (A), A/Duck/Czechoslovakia/56(H4N6) (B) and A/Swine/Hong Kong/9/98(H9N2) (C). Positions of the gel elements with immobilized oligonucleotides are as shown in Figure 1.

signals register in the d1–d5 group of elements only, while all other signals are close to the background level. This result indicates that the sample belongs to the H5 subtype. Within the NA cluster, only h1–h5 elements produce positive signals, where the probes for the N1 subtype are grouped together. Other positive field samples produced similar hybridization patterns. Thus, the strain that caused the outbreak was identified as the H5N1 subtype. The isolate presented in Figure 4 was defined as A/Duck/Novosibirsk/56/05(H5N1) (DQ230522).

The analytical sensitivity of the developed procedure relative to virus titer (detectable by standard virus isolation in chicken embryos) was evaluated using A/WSN/33(H1N1) and A/Victoria/3/75(H3N2) reference strains as described in Materials and methods. The lowest viral concentration at which all samples from both isolates were confidently detected was determined to be 10^3 EID_{50}/reaction. Few samples were positive at lesser concentrations with the lowest equal to 10^2 EID_{50}/reaction.

Table 3. Comparison of identification results obtained with microarray analysis and 'gold standard' immunological subtyping for 41 field samples

On-chip hybridization result	Immunological subtyping			Specificity (%)	Sensitivity (%)	Positive predictive value (%)	Negative predictive value (%)	Efficiency (%)
	Positive H5	Negative	Total					
Positive H5N1	16	0	16	100	76	100	80	88
Negative	5	20	25					
Total	21	20	41					

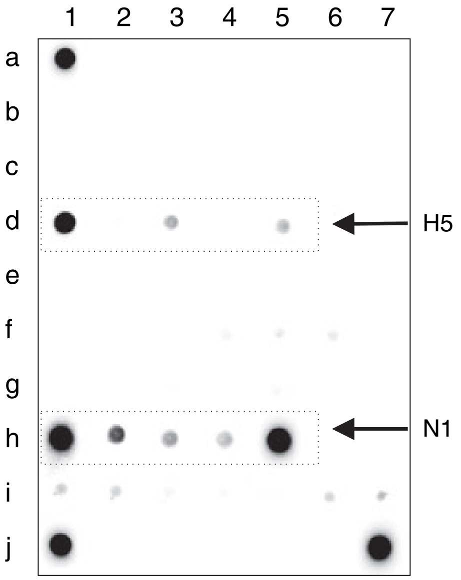

Figure 4. Example of hybridization pattern of the field specimen identified as H5N1. RNA was obtained from a suspension of internal organs of a domestic duck which died during an outbreak of influenza in the Novosibirsk region in 2005.

In order to evaluate reproducibility of the method, RNA was isolated from cultivated A/Sydney/5/97(H3N2) strain in five independent assays, and the fragments of HA and NA genes were amplified and then hybridized on microchip. The maximal deviation of measured fluorescence intensities from the average values was no more than 20% (Figure 5).

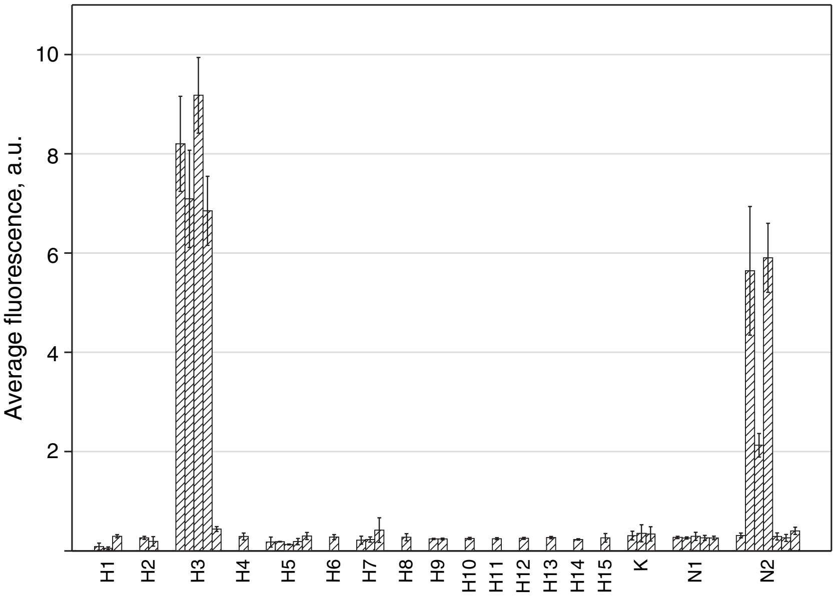

Figure 5. Relative values of fluorescent intensities (arbitrary unit) from the each gel pad averaged among five assays for reference H3N2 subtype. Each column represents the mean value of fluorescent intensity obtained for the defined gel pad. Error bars show the standard deviations of measured fluorescence intensities from mean values.

Discussion

As discussed in an earlier review, biochips developed in our group and consisting of 3D hydrogel elements offer significant advantages over widely used 2D microchips.[22] In particular, three-dimensional gel networks possess a higher capacity for immobilization of probes with respect to immobilization on a two-dimensional surface. This implies higher intensity of fluorescent signals and suppresses statistical deviations both within the same biochip and between different biochips compared with 2D chips.

Using the technological platform of gel-based biochips, we developed a fast and efficient diagnostic complex consisting of affordable equipment and matching software with the sensitivity of 1–5 amol of fluorescence dye per gel element.[23]

This complex of biochips, equipment, and software is already in use in many biomedical applications for the analysis of bacterial and viral genomes, including the identification of species of orthopoxviruses – known pathogens for animals and humans, as well as old samples of smallpox,[24] and identification of mutations of HIV which confer resistance to protease inhibitors.[25] Diagnostic biochips for the identification of drug-resistant tuberculosis[20,26] have been approved for clinical application by the Ministry of Health of the Russian Federation.

Gel-based biochips for the subtyping of influenza virus A can be used in clinical laboratories for diagnostics and – on a larger scale – for the monitoring of epidemiological situation. Such monitoring of different influenza A subtypes is especially important for commercial poultry operators.

The approach to subtyping described here is simple, reliable, and quick: the results can be obtained within 10 hours. This time include stages of RNA preparation (1.5 hours), amplification (1.5 and 1 hour), hybridization (5.5 hours), and washing (10 minutes). In comparison with traditional methods of virology, our approach is faster and does not require any live systems for cultivation.

We tested our method using 21 reference strains of influenza A virus representing nine different subtypes of the HA gene (H1, H2, H3, H4, H5, H7, H9, H10, and H13). Currently, we have two groups of NA-related oligonucleotides included in the call set of the biochip. They identify the two subtypes N1 and N2, which were chosen because they are common both for circulating human flu strains (H1N1, H3N2) and the highly pathogenic avian strain H5N1. We are planning to include more oligonucleotides to identify additional subtypes of NA. Of the 21 tested strains, 12 belong to either N1 or N2 subtypes, while the other eight strains belong to subtypes N3, N6, N7, N8 and N9 as was determined by immunological methods. Multiplex PCR with subsequent hybridization with the biochip described here enabled us to identify simultaneously the subtypes of HA and NA. The specificity of the method with previously cultivated strains was 100%, and no false positives were observed.

As for cloacal swabs obtained in infected area from fowls with no clinical symptoms, only 8 out of 33 were positive when tested on the biochip compared with 13 positive found using the traditional immunological approach. These false-negative failures resulted from the amplification step and not from any limitations of the microchip. Nevertheless the field specimens taken from sick and dead birds (pooled organ specimens) were successfully identified by the biochip, and the results were in full agreement with those obtained by the traditional immunological methods.

The assay described here showed the analytical sensitivity equal to 10^3 EID_{50}/reaction; also few successful results with 10^2 EID_{50}/reaction were taken. It is 10–100-fold less sensitive then conventional RT-PCR[9,11] and no match for even more sensitive real-time PCR assays.[8,12] A comparison of the sensitivity between the different assays utilizing microchip technology is difficult because of the use of different viral strains and viral concentration methods. In addition the proposed techniques used only previously isolated viruses as a starting material for RNA extraction. The distinction in sensitivity of our method from conventional RT-PCR is related to the extreme variability of the target HA and NA genes. The lack of mutual conservative regions within different subtypes results in limited opportunities for designing primers. Use of the multiplex primer set to produce both HA and NA gene fragments may also complicate the amplification step, thus decreasing the sensitivity. Despite the success in subtyping of influenza virus in viral isolates and field specimens from sick and dead birds, further improvement in target gene amplification is needed. At present, we are attempting to develop a novel amplification technique with the required sensitivity.

Finally, the results indicate that the method discussed in this study has a good reproducibility, as shown by a low deviation from mean values of measured fluorescence intensities among performed assays.

We conclude that the method of subtyping using gel-based biochips is a promising approach to fast detection and identification of influenza A virus in humans and animals with symptoms of acute infection. Broad application of this approach may improve our understanding of the emergence and course of influenza outbreaks and provide timely warning of dangerous epidemiological situations. In the future, biochips may become a viable technological platform in the clinical diagnosis of influenza in both humans and animals.

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