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.

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 Thailand⁴ 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. 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, isothermal PCR, or a combination thereof. 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. A more advanced microchip developed recently by Lodes et al. 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. Strains were used when their titer was at least 10⁶ units per ml of 50% egg infective dose (EID₅₀).

For analytical sensitivity studies human viral isolates A/WSN/33(H1N1) and A/Victoria/3/75(H3N2) with known EID₅₀/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 EID₅₀ 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

| 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          |
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 TT-3′ and F552na 5′-TGG GTT TGC AGA GAT
AAT TGG-3′. Twentyfive microliters of the reaction mix
contained 2.4 mm MgCl2, 80 mm KCl, 16 mm Tris–HCl,
P H 9.0, 0.2 mm of each dNTP, 5 U of Taq DNA polymer-
ase, 25 U of M-MLV reverse transcriptase (both from Sil-
ks, 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 ‘Ter-
cyc’ (DNA-Technology, Moscow, Russia). The reaction mix-
ture 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 seg-
ment 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′-TTTGAATTCTACCA-
CAAGTGTGA-3′) for HA gene and R556na* and F551na
(5′-ATGGTGTGATGGAGAAGAC-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 reac-
tion mixture contained in 25 μL 70 mm Tris–HCl, pH 8.6,
16.6 mm (NH4)2SO4, 2.5 mm MgCl2, 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 subse-
quent gel immobilization, a free amino group was intro-
duced 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 accord-
 ing 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 manufac-
tured as described earlier.19

Hybridization
Hybridization was carried out by adding 12 μL of the reac-
tion mixture obtained after the second round of amplifica-
tion 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 minutes 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 influ-
enza. The call set of the oligonucleotides identifies 15 sub-
types 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 per-
formed 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, H11, H12, and
H13 (Figure 2). H11 and H12 are complementary to two
variants of the subtype H1 and are separated by 27 nucle-
tides. At the same time, H12 and H13 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.
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.\textsuperscript{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

| 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 TCC AAC | 1584–1604 | CY015580 |
| H1.3              | GAT TCT GGC GAT CTA TTT AAC | 1584–1604 | CY015580 |
| H2.1              | AAA TTT AGC ACT AGT GGG GTT | 1592–1612 | CY015135 |
| H2.2              | AAA TTT AGC AAY ATG GGG GTT | 1592–1612 | CY015135 |
| H3.1              | TGY TTT TGG TTT TGG TGT GGT | 1626–1645 | CY016108 |
| H3.2              | CTY TTT GCC ATA TCA TG | 1608–1627 | CY016108 |
| H3.3              | GAA GCA TTA AAC AAC CGG TT | 1536–1555 | CY016108 |
| H3.4              | ATT TCC TCC GCC ATA TCA TG | 1608–1627 | CY016108 |
| H3/H4             | ATT TCA TCC GCC ATA TCA TG | 1608–1627 | CY016108 |
| H4                | CTA ACA TGT GGG AGT TCC | 1617–1634 | AF046080 |
| H5.2              | TCT ACG TGT GCC AGT TCC | 1617–1634 | AF046080 |
| H5.4              | CAA TGG GAA CTT ACC AAA TAC | 1585–1605 | AF046080 |
| H5.5              | CAT GGT AGT TGG TCT TGC 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 | 1543–1556 | CY015014 |
| H7.2              | CAA TGC ARA ATA GAA TAC AAG TTA GAG | 1547–1571 | CY015014 |
| H7/H10/H15        | TTT AGC TTC GGG GCA TCA TGT | 1615–1635 | CY015014 |
| H8                | GCC GGC CAG TCT TTG CTT | 1608–1625 | D90304 |
| H9.1              | TGT CTC TCT ATC TCT TTT G | 1590–1608 | AB256706 |
| H9.2              | TCG GCC GGA ATG ATC CCT CAA 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 GGA GTG TTG CC | 1608–1627 | CY014598 |
| H13               | AAC GTT TAC AAA GCA TTR TC | 1617–1636 | M26091 |
| H14               | CTT TGT CCT GGT GCC ACT GAT T | 1643–1664 | CY014604 |
| H15               | ATT AGG ATA ATG ATC AAT C | 1573–1591 | CY006032 |
| N1.1              | TTT GAR ATT TGG GGT GAT CC | 1131–1150 | DQ376693 |
| N1.2              | TTT GAR ATG TGG GAT 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 GTC | 1221–1240 | DQ376693 |
| N1.5              | GGA TAT AGC GGG AGT TTT GT | 1221–1240 | DQ376693 |
| N2.1              | TGY TTT AAY AGG TGT TTT TAT G | 1253–1274 | CY016118 |
| N2.2              | TGY TTT AAT MGG TGG 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 AAA 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 AGT CAT | 1174–1194 | CY016118 |

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

**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.
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.

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).

**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. 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

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

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

![Figure 4](image-url) Example of hybridization pattern of the field specimen identified as H5N1. RNA was obtained from a suspension of inner organs of a domestic duck which died during an outbreak of influenza in the Novosibirsk region in 2005.

![Figure 5](image-url) 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.
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 tuberculosis26 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 $10^7$ EID$_{50}$/reaction; also few successful results with $10^5$ EID$_{50}$/reaction were taken. It is 10–100-fold less sensitive than conventional RT-PCR$^8,11$ 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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