Oligonucleotide microarray for subtyping of influenza A viruses

Klotchenko S.A.1, Vasin A.V.1, Sandybaev N.T.2, Plotnikova M.A.1, Chervyakova O.V.2, Smirnova E.A.1, Kushnareva E.V.1, Strochkov V.M.2, Taylakova E.T.2, Egorov V.V.1, Koshemetov J.K.2, O.I. Kiselev1, A.R. Sansyzbay2

1 Research Institute of Influenza of the Ministry of Health and Social Development of the Russian Federation, 15/17 Prof. Popova St., St. Petersburg, Russia,
2Research Research Institute for Biological Safety Problems of the RK NBC/SC ME&S RK, Gvardeisky, Republic of Kazakhstan

E-mail: vasin@influenza.spb.ru

Abstract. Influenza is one of the most widespread respiratory viral diseases, infecting humans, horses, pigs, poultry and some other animal populations. Influenza A viruses (IAV) are classified into subtypes on the basis of the surface hemagglutinin (H1 to H16) and neuraminidase (N1 to N9) glycoproteins. The correct determination of IAV subtype is necessary for clinical and epidemiological studies. In this article we propose an oligonucleotide microarray for subtyping of IAV using universal one-step multisegment RT-PCR fluorescent labeling of viral gene segments. It showed to be an advanced approach for fast detection and identification of IAV.

1. Introduction
Influenza is an acute respiratory disease bringing on annual seasonal epidemics and irregular hardly predictable pandemic outbreaks. The etiological agents of influenza are the viruses of the same name, the members of Orthomyxoviridae family. Among the three known types (A, B and C) of influenza viruses the most serious infectious threats arise from influenza A viruses (IAV) due to their genetic variability and capacity for a rapid and global spread. IAV are classified into subtypes on the basis of the serological differences of two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). IAV infect humans, birds, pigs, horses and marine mammals, the natural reservoirs of IAV are waterfowls. 16 subtypes of HA and 9 subtypes of NA have been described so far. In human population only three HA (H1, H2, H3) and two NA (N1, N2) subtypes predominate, but the infections of humans with high pathogenic IAV strains, such as H5N1, H7N7 and H9N2, have been recently detected. Moreover, the reassortment of IAV segments can lead to the emergence of new virus variants, such as for example «Spanish flu» virus in 1918 or swine-origin A/H1N1 virus in 2009.

Since the pandemic potential of IAV is associated with the spread of new atypical virus subtypes, the development of rapid and precise diagnostic methods of IAV detection and subtyping is an extremely urgent problem of influenza surveillance and epidemiology. One of the most promising technologies for IAV subtyping is microarray approach, the number of which have been developed recently [1-7]. Earlier we offered the method of IAV genomic RNA amplification using universal primers for application in influenza subtyping microarrays. This pair of primers to the conservative
terminal regions of all IAV segments greatly unifies influenza microarray systems making them independent from the complex set of multiplex primers specific for certain IAV subtypes. In current paper we propose universal IAV subtyping oligonucleotide microarray using the approach described above.

2. Materials and methods
IAV strains were obtained from the collection of Research Institute of Influenza of the Ministry of Health and Social Development of the Russian Federation.

2.1. RNA isolation.
Total RNA was isolated from the allantoic fluid using TRIZol reagent (Invitrogen, USA). RNA concentration and integrity were measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA).

2.2. Microarray design.
Oligonucleotide probes, containing 5'-aminolinker, were synthesized and HPLC purified by «DNA-synthesis» (Russia). Probes (300 ng/µl) in 3×SSC were immobilized on Vantage Aldehyde Slides (CEL Associates, Estonia) using SpotBot 3 Personal Microarrayer (ArrayIt, USA). After printing slides were treated with UV (0.09 J/cm²). Before hybridization slides were boiled in miliQ H2O for 1 min.

2.3. Fluorescent labelling of IAV genomic segments.
Fluorescent labelling of IAV cDNA was performed in the process of the direct incorporation of Cy3-dCTP by multisegment RT-PCR using SuperScript III One-Step Quantitative RT-PCR System (Invitrogen, USA) with universal MBTuni-12 primers [8,9] as described earlier [10]. The reaction mixture additionally contained 20 µl 1mM Cy5-dCTP. Amplification conditions for Rotor-Gene 6000 thermocycler (Corbett Research, Australia) were as follows: 1) 45°C for 60 min; 2) 94°C for 2 min; 3) 5 cycles at 94°C for 30 sec, 45°C for 30 sec and 68°C for 3 min; 4) 31 cycles at 94°C for 30 sec, 57°C for 30 sec and 68°C for 3 min.

2.4. Hybridization.
30 µl of fluorescently labeled cDNA was incubated at 99°C for 2 min, chilled on ice for 2 min, afterwards 10 µl of 99% formamide and 10 µl of 5× hybridization buffer were added. Hybridization was performed in 50 µl using «Whatman» frames at 37°C for 2 hours with agitation at 250 rpm. After hybridization slides were washed in the mixture of 1×SSC buffer and 0.1% SDS.

2.5. Microarray scanning.
After hybridization slides were scanned using ScanArray Express (“PerkinElmer”, USA) with 5 µm resolution. Data analysis was made using ScanArray software.

3. Results and discussion
The selection of IAV subtype-specific oligonucleotide probes was made by the multiple alignments of more than 15 000 HA and 16 000 NA sequences deposited in GenBank. On the basis of the alignments, made using MAFFT software (http://mafft.cbrc.jp/alignment/server/), sequences were divided into separate groups, for which specific oligonucleotide probes were designed using OligoWiz software (See tables 1A,B,C). It was not always possible to select the unique probe for each HA or NA subtype, for this reason several probes were selected for some subtypes. Oligonucleotide probes were synthesized and immobilized on microarray slides using contact printing method. IAVmicroarray consisted of 16 identical subarrays, containing 20 rows, corresponding to 16 probes for HA (H1-H16), 9 probes for NA (N1-N9) and 7 control probes for NP.
and M segments (See figure 1). The diameter of each spot was 200 nm, all probes were immobilized in 3 repeats.

Table 1A. Oligonucleotide probes for IAV hemagglutinin subtyping.

| Probe | Oligonucleotide sequence (5' → 3') | Length, n. |
|-------|------------------------------------|------------|
| H1-1  | GAACGGAGAATGAACTATTACTGGGACTAGTGAAGCAGGGAGACAA | 47         |
| H1-2  | GTCTTCCCTGGGGCGACATCAGTTTCTGAGTGTYCTAATGGG | 42         |
| H1-3  | ACAGGGCTGCAAGCTTCCCACTCCATCAATCCAGAGGTTTGGTTG | 47         |
| H2-1  | TAAAGAATGTTCCTCCAGATGAACTAGAAGAGGATTTGTTGGGGCAAT | 46         |
| H2-2  | GTCAACCTGTACCTGACAGGACATCTTTGGAGAAAGCAATAATG | 47         |
| H2-3  | TATGCTCAGTAGCAGGGTTCCTGCTGACTGCAATCATGATA | 42         |
| H2-4  | TGGGATGTGCATAATTTTGAGACACTGTTGAATTATATGCCACGAAATA | 50         |
| H3-1  | ATGTGGGCTGCACARAGGGCAACATTAGTGGCAACATTTGTC | 42         |
| H3-2  | ATGTGGGGCTGCCAAAAGGGCACACAGATGGCAACATTTGTC | 42         |
| H3-3  | CACAACTCTGATTCATATATGATAATGGGATTATGACATGCT | 42         |
| H3-4  | CCCAAGGGTCACATGCTTCTGACACTCCTTTTAGG | 47         |
| H3-5  | TTTATAGGGGAATGGGCAAGGAAAATGAGATGTTGGTAT | 44         |
| H3-6  | CATAGAATGCTGACTTAAATAGTGAACACTTCCTGTCATGCAAATGGAAT | 50         |
| H3-7  | CTTTTCTGGTCACATGCTCTTTTATAGTGAACCTTTTAGC | 47         |
| H3-8  | CAAAATCCCTGGCTTTATATGACGAGATCGTACGTTGGT | 42         |
| H3-9  | CCATGGGGTTGATGTTCAATGATTGCACATGCA | 43         |
| H7-1  | ATGGGGCTGTITTTTCTTCAATAAGAAGAATGAAAATGAGCRGTGCATAT | 50         |
| H7-2  | CTTCGGGCCATCAGTTTCATTCATCTTGGGCAATGAAATGGG | 42         |
| H7-3  | CTCAAAATGCAAGGAGAAARTGGCAGAAACTGTCGAGTCACA | 42         |
| H7-4  | TGGTATAGCTTCGGGACATCAGTTCTTTCTGACATGCAAATGGAAT | 42         |
| H8    | ATTTACAGTACGATGGCCGAGTCTYCTGTCGTGCAAATCTC | 42         |
| H9-1  | CTTAAGGAATCTCYACATTATTTTCGACTGCGGCTCACTTCCTTGT | 46         |
| H9-2  | CCAATGGGTTGCTGCCTCTYCTTCATTTGGGCAATGCAAATGGAAT | 42         |
| H9-3  | TTTGCCCCAGTGCTGCAATGCTTCGAGATGCAAATTTGGT | 44         |
| H10-1 | CTTTTGTGCTGTATCATGAGGGCTTTGTTTCTCTCTGTGTCAAATGGAAT | 50         |
| H10-2 | GTCAATCATGGACAGATTCCACATACCTGGGACTACATTACG | 49         |
| H11-1 | GATCTCCATGATTCATGATGACGAAACCTCAGTAAAAGGGTCAAGAATG | 42         |
| H11-2 | TGGGATGTGCAGAATGATGAGGTATGAGATGATGAGAGT | 42         |
| H12   | TACTGCTCATGATTATTTGGGGCTTCTTTTCTCGGRTGCATAAATGGAAT | 50         |
| H13-1 | ATGTGGGGCTGTITTTTCTTCAATAAGAAGAATGAAAATGAGCRGTGCATAT | 50         |
| H13-2 | ATAAATAGTTGCTGACAGAAATAGATGAYGCTGAATACGTAGTGA | 45         |
| H14   | TGCATACCAGCATAAACGCATAATGAGACCAAAAGGCAATCTTACAA | 45         |
| H15   | GCTGATCTGATAATAGAAGAAGAATTCAGATGACATCTGTTACACCAG | 50         |
| H16-1 | TAAATGCAATGAGAAGAATGAGGCTTCTCAATCTCTTCTTCAACA | 45         |
| H16-2 | ATTCGAATGTCGACATCATAATCAGAGACTACAAAGAGAGGTCACA | 48         |
**Table 1B.** Oligonucleotide probes for IAV neuraminidase subtyping

| Probe | Oligonucleotide sequence (5’ → 3’)$^a$ | Length, n. |
|-------|---------------------------------------|------------|
| N1-1  | GGGTTGCTTGGCAGACGGTGCTGAGTGGCATAITTYACCATT | 43         |
| N1-2  | TCCTAATGGGACARATACCCGAAGTCATTCTCTAGTGGAAACAGGATT | 50         |
| N1-3  | TGGTCTTGGGACAGCAGGGCAGGTGGAGTTGCCTACCATTTGAC | 42         |
| N1-4  | CAAGAGTCTGAACTGCTGATGTAATGYYCCTTTACTGTAATGAC | 42         |
| N2-1  | CAAGTGTGCACTGACTGAGCATAGCATGGTCAGCTCAAG | 44         |
| N2-2  | TTGGSRACAAACAAAGGTCATAGCATGGATCAAGGACAATGGAACAGGCTCAGTCGTCGATT | 42         |
| N2-3  | TTTGTTGCCACCCAGYACHTATAGGAAACCAGGGCTCATGGGCTGAT | 42         |
| N2-4  | TGTGHAATGCATATGGAACAGGCTCAGTGCGCCTGAT | 42         |
| N3    | AGYAAATGATAATGCCCTTGGTAAGYTAAGCACACTGGATTTGAGGA | 50         |
| N4    | TGGTTGTTTAATGCAATACCCAAAGGTGTCATGGCCTGGATATCCGATTCGAT | 45         |
| N5    | TTTTGTGCTTCTGACTGAGCATAGCATGGTCAGCTCAAG | 42         |
| N6-1  | TATCGCCATGACGCGCATCTCAGAATATTCRATCATGCTCAGTCGA | 42         |
| N6-2  | GACGCCATGACTGTCATGAGTTCATGCTGATGTCAGTARATC | 42         |
| N6-3  | GAGCGATTGGGATCGTGGTCATGAGCATTGAGGCTGAAATTC | 42         |
| N7-1  | GTTGAAGGATGGGTAGTGGTGGYACAAGGACAATGCCATAAAG | 44         |
| N7-2  | CAGTTGCCGCGTTCCTCTCCCAGATGGGACARACTCACAAT | 42         |
| N8-1  | ATATTGGGACCTCAGGATGCTGATGTTGAGTGAGTAYCATT | 45         |
| N8-2  | TGGTGTAGAGAATACGGGCCAATCCACCAATGGTGACCGAGCAGTGGTACGGTTTGCAGATCGAT | 45         |
| N8-3  | TGGTGTAGAGAATACGGGCCAATCCACCAATGGTGACCGAGCAGTGGTACGGTTTGCAGATCGAT | 42         |
| N9    | ATGTGTTCCAGYACAGAAATCCCTGGACGCTGGCCTGAT | 45         |

*Apart from the four (A, G, C, T) bases, abbreviation codes are used: Y denotes C or T; R – A or G; W – A or T; S – G or C; M – A or C; K – G or T; V – A or C or G; H – A or C or T.

For the validation of IAV subtyping microarray we used different IAV strains. Total RNA from IAV samples was isolated using TRIZol reagent. Full-length cDNAs, corresponding to IAV genes, were amplified using universal MBTuni-12 primers in the process of the RT-PCR with Cy3-dCTP as suggested and described earlier [10]. The Cy3-labeled IAV cDNA samples were denaturated and further used for hybridization with microarray slides during 2 hours at 37°C.

First of all we used entirely characterized strains A/Vic/3/75(H3N2), A/chicken/Kurgan/5/05 (H5N1), A/Brisbane/59/H1N1), A/swine/1976/31/H1N1), A/Brisbane/10/07(H3N2), A/California/07/09(H1N1). Data presented on figures 2 and 3 showed the specific binding of viral cDNAs with the probes, immobilized on the microarray slide. All IAV strains were subtyped correctly.

After that we used two partly characterized IAV strains of H2N2 and H1N1 subtype and four IAV strains, for which only HA subtype was determined (A/H6_51c, A/H3_402, A/H3_111 and A/H9_121). Significant levels of fluorescence were observed only for probes, specific to the corresponding IAV subtypes, for all other oligonucleotides fluorescence intensities were on the approximately equal level and comparable with standard error (See figure 3). NA subtypes were

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**Table 1C.** Oligonucleotide probes for IAV typing

| Probe | Oligonucleotide sequence (5’ → 3’)$^a$ | Length, n. |
|-------|---------------------------------------|------------|
| NP-1  | ACGAAAGGGCAAGCCGATCTGCTTCTCGACATGA | 42         |
| NP-2  | ATGAGTTAATGAGGGCATTTTCTTCGAGACATGCAARGAG | 45         |
| M2-1  | GCAGARTGCTGTGGATGTTGACGATRGTCATTTTTGCTCAACTAT | 44         |
| M2-2  | CCTACAGAAACAGATGCGGTGAGCTGACATCGAACTAGTA | 44         |
| M2-3  | CCTCTACAGAAGGGATRGCCAGTCTATAGGGAAGATATCG | 44         |

*Apart from the four (A, G, C, T) bases, abbreviation codes are used: Y denotes C or T; R – A or G; W – A or T; S – G or C; M – A or C; K – G or T; V – A or C or G; H – A or C or T.
unambiguously determined: N1 for A/H6_51c strain, N8 for A/H3_402 and A/H3_111 strains and N7 for A/H9_121.

Figure 1. Layout of IAV subtyping microarray. H – hemagglutinin, N – neuraminidase, PC – positive control

Finally two IAV strains of the unknown subtype were analyzed. The subtype of these IAV strains could not be determined by the traditional methods. We easily determined these strains as A/H5N1 and A/H3N8 using IAV subtyping microarray.

Figure 2. The fluorescence intensities of IAV subtyping microarray after hybridization with Cy5-cDNA, obtained from the (1) A/Vic/3/75(H3N2), (2) A/chicken/Kurgan/5/05(H5N1), (3) A/Brisbane/59(H1N1), (4) A/swine/1976/31(H1N1), (5) A/Brisbane/10/07(H3N2) and (6) A/California/07/09(H1N1) virus strains.

The developed method for identification and subtyping of IAV via hybridization on a microarray slide showed good results. All the studied samples were correctly classified into subtypes according to the fluorescent signals from capture probes. We did not observe significant cross-reactivity by capture probes. Currently developed oligonucleotide IAV subtyping microarray in combination with multisegment RT-PCR method for amplification of fluorescently labeled cDNA of all possible IAV subtypes with a single pair of primers is a very promising approach for rapid and accurate...
identification of all subtypes of IAV. Moreover, it allows molecular screening not only of HA and NA, but of all other IAV genes.

**Figure 3.** Relative values of the spot fluorescence intensities, derived after hybridization with reference IAV cDNA samples. Each column corresponds to the 3 spots mean intensity.
4. References

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