Multisegment one-step RT-PCR fluorescent labeling of influenza A virus genome for use in diagnostic microarray applications

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Abstract. Microarray technology is one of the most challenging methods of influenza A virus subtyping, which is based on the antigenic properties of viral surface glycoproteins – hemagglutinin and neuraminidase. On the example of biochip for detection of influenza A/H5N1 virus we showed the possibility of using multisegment RTPCR method for amplification of fluorescently labeled cDNA of all possible influenza A virus subtypes with a single pair of primers in influenza diagnostic microarrays.

1. Introduction.
The subtyping of influenza A viruses (IAV) is based on the antigenic properties of two surface glycoproteins – hemagglutinin (HA) and neuraminidase (NA). IAV infect several animal species including humans, birds, pigs, horses and marine mammals. 16 subtypes of HA and 9 subtypes of NA have been described so far. In human population only three HA subtypes (H1, H2, H3) and two NA (N1, N2) subtypes predominate, but infections of humans with high pathogenic IAV strains, such as H5N1, H7N7 and H9N2, have been recently detected. Moreover, reassortment of viral segments can lead to the emergence of new virus variants, such as vH1N1 in 2009. For these reasons the development of new diagnostic methods for detection and subtyping of all existing and plausible new IAV strains is an extremely urgent problem of influenza surveillance [1].

Commonly used PCR methods are usually subtype specific and do not provide information about other subtypes. Recently the number of biochips for detection of 16 known subtypes of HA and 9 subtypes of NA have been developed [2,3], but one of the main problems of IAV microarray detection is associated with the choice of cDNA fluorescent labeling methods. In this article we propose the amplification of genomic RNA segments using universal primers to the conservative terminal regions of all IAV genomic segments for use in influenza subtyping microarrays. The application of these primers makes it possible to unify influenza microarray systems and to get rid of the complex set of multiplex primers specific for certain IAV subtypes.
2. Materials and methods.

2.1. RNA isolation
Total RNA was isolated from the allantoic fluid using TRIZol reagent (Invitrogen). RNA concentration and integrity were measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). Influenza virus A/H5N1 strains were 6:2 A/PR/8/34(H1N1) reassortants, obtained in St. Petersburg Influenza Institute using reverse genetics.

2.2. Oligonucleotide microarray design and printing
Oligonucleotides containing 5'-aminolinker were synthesized and HPLC purified by «DNA-synthesis» (Russia). The probes (300 ng/µl) in 3×SSC were immobilized on Vantage Aldehyde Slides «CEL Associates» using SpotBot 3 Personal Microarrayer («ArrayIt corporation», USA). After printing the slides were treated with UV (0.09 J/cm^2). Before hybridization the slides were boiled in miliQ H_2O for 1 min.

2.3. Fluorescent labeling of IAV genomic segments
Fluorescent labeling of cDNA was performed in the process of direct incorporation of Cy3-dCTP with M-RTPCR using SuperScript III One-Step Quantitative RT-PCR System (Invitrogen, USA) with universal MBTuni-12 (5'-ACGCCGTGATCAGCAAAAGCAGG-3') и MBTuni-13 (5'-ACGCCGTGATCAGTAGAAACAAGG-3') primers [4]. The reaction mixture additionally contained 20 µl 1mM Cy5-dCTP. Amplification conditions for Rotor-Gene 6000 thermocycler (Corbett Research, Australia) were the following: 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 the slides were washed in the mixture of 1×SSC buffer and 0.1% SDS.

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

3. Results and discussion.
For estimation of possibility of using fluorescently labeled viral cDNA in the process of M-RTPCR for IAV subtyping microarrays we developed the test biochip for human A/H5N1 detection. 50-mer oligonucleotide probes, complementary to HA and NA cDNAs of human A/H5N1 strains deposited in GenBank database, were designed using OligoWiz software [5]. Multiple alignment of these HA and NA sequences did not reveal any conservative 40-60-mer regions, which could be used as universal targets. For this reason HA sequences were divided into two and NA – into three groups, for which specific oligonucleotide probes denoted as H5-I^H^N^1^, H5-II^H^N^1^, N1-I^H^N^1^, N1-II^H^S^N^1^ and N1-III^H^N^1^ were designed (See figure 1). The biochip consisted of 16 identical subarrays containing 11 rows corresponding to 5 probes for A/H5N1, 4 probes for A/H3N2 and A/H1N1 reference strains detection (H3^H^N^2^, N2^H^N^2^, H1^H^N^1^, N1^H^N^1^) and 2 negative controls. The spot diameter was 200 nm, all the probes were immobilized in 10 repeats (See figure 2).
For viral cDNA fluorescent labeling one-step M-RTPCR method with universal primers for terminal regions of IAV gene segments was used. The incorporation of fluorescently labeled dNTP (Cy5-dCTP) into the reaction mixture changes the conditions of reaction dramatically. For this reason optimal Cy5-dCTP concentration (20 µM) and amplification conditions were evaluated on the basis of amplicons analysis. The results of hybridization of the fluorescently labeled cDNA samples of A/Kurgan/05/2005(H5N1), A/Moscow/10/99(H3N2) and A/New Caledonia/10/99(H1N1) (See figure 3) showed that they had specifically binded with the probes on the biochip. Significant levels of fluorescence were observed only in the 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 4). The median values of the spot fluorescence intensities after deduction of fluorescence background were used as significant fluorescence values.
Figure 2. Layout of influenza A/H5N1 subtyping biochip. NC – negative control

Moreover, for the analysis of specificity of the suggested approach we used A/chicken/Astana/6/2005(H5N1), A/duck/Singapore/3/1997(H5N3), A/VietNam/1203/2004 (H5N1) and A/Texas/01/2009(H1N1) strains. The data presented on figure 5 showed no nonspecific binding: only human and bird A/H5N1 virus strains were detected, A/duck/Singapore/3/1997 strain of A/H5N3 influenza virus subtype was not detected. Although «Swine» A/Texas/01/2009 (vH1N1) virus nonspecifically binds with N1-I^{vH1N1} probe, the level of binding is comparatively low.
Figure 5. Graphical presentation of spot fluorescence intensities after hybridization with A/chicken/Kurgan/5/2005 (H5N1), A/VietNam/1203/2004 (H5N1), A/Texas/01/2009 (H1N1), A/chicken/Astana/6/2005(H5N1), A/duck/Singapore/3/1997(H5N3), A/VietNam/1203/2004 (H5N1) and A/Texas/01/2009(H1N1) virus samples

On the example of microchip for detection of influenza A/H5N1 virus we showed the possibility of using multisegment RTPCR method for amplification of fluorescently labeled cDNA of all possible influenza A virus subtypes with a single pair of primers in influenza diagnostic microarrays. Moreover, it allows molecular screening not only of HA and NA, but of all other IAV genes.

4. References

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