

# Development of the cell-ELISA test for the subtype identification of circulating influenza A(H1) and A(H3) viruses

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## ABSTRACT

The sensitive version of cell-ELISA was developed for the subtype-specific differentiation of current influenza A(H1N1)pdm09 and A(H3N2) viruses that are circulating in the human population. This method is based on the estimation of virus reproduction in infected MDCK cells. The detection step of this method is an interaction of the subtype-specific monoclonal antibodies (mAbs) with the viral hemagglutinin (HA) molecule. The influenza A virus strains, isolated in the 2014 epidemic season, were used to validate this method.

It was shown that when using mAbs # 1/ # 2 or # 4 at a concentration of 10-15 µg/ml, the developed variant of cell-ELISA was able to detect HA protein synthesized in the infected cells of influenza A(H3N2) and A(H1N1)pdm09 viruses, respectively.

The developed method can be used for the identification of modern influenza A viruses with low hemagglutination activity, which is not possible by the conventional hemagglutination inhibition test.

## INTRODUCTION

Influenza is one of the most widespread infectious diseases. Around 50 million infectious disease cases are recorded annually in Russia. Severe acute respiratory illnesses (SARI), including influenza comprise 95% of all recorded cases. The influenza morbidity among the children is 7 to 10 times higher than that among the adult population. According to the data of the Federal Influenza and SARI Centers, the number of morbidity and mortality cases increases significantly during the influenza pandemic compared to the annual epidemics [1].

The high probability of the emergence of new pathogens, including the potential pandemic variants of the influenza viruses, is recognized by the World Health Organization (WHO) as an important reason for the continuous improvement of the respiratory virus surveillance service. This is particularly important in order to recognize forthcoming pandemics and to identify new influenza viruses as early as possible [2].

Since the SARI of a different etiology often have very close clinical symptoms, especially in the beginning of the disease, having a proper diagnostic becomes especially important. The early identification of the influenza infection makes it possible to prescribe the corresponding treatment to the patient at the very beginning of the illness in order to prevent the development of other secondary infections and limit the use of antibiotics. The timely determination of the nature of epidemic outbreaks is necessary to determine the tactics for conducting the anti-epidemic campaign and to plan the urgent preventive measures. In this

connection, the optimization and development of the effective diagnostics of influenza and other SARI is an important issue.

The most common method of identification of the newly isolated influenza viruses is the hemagglutination inhibition assay (HAI) with the use of specific immune animal sera. This method allows for the determination of the specificity of the antigen – antibody interaction. However, the continuous accumulation of the evolutionary mutations in the hemagglutinin (HA) molecule of influenza A(H1N1) and especially A(H3N2) strains during the last decade have brought changes to the HA receptor-binding site that in turn led to the reduction of HA affinity to the receptors of different types of erythrocytes [3-6]. This effect complicates the identification of the subtype of some modern virus strains by HAI test.

One of the alternative methods of the identification of modern influenza viruses is micro cell-ELISA. This method is based on the estimation of the virus reproduction in infected cells by using high specific monoclonal antibodies (mAbs) that interact with the virus proteins. Cell-ELISA with the use of specific mAb to the virus NP protein was recommended by the WHO for the measurement of virus neutralizing antibodies in a microneutralization assay [7]. This method is also successfully used for the detection of the virus strains that react poorly in HAI [8].

Since the structure of NP protein is highly conserved for influenza viruses of different subtypes, cell-ELISA with mAb specific to the NP protein allows for the

detection of all subtypes of the influenza virus A strains, but it does not allow for differentiating the virus subtype.

The goal of this project was to develop a sensitive version of the cell-ELISA that is suitable for the identification of the subtype of modern influenza A(H1N1)pdm09 and A(H3N2) strains with the use of a subtype specific mAbs to the viral HA.

## MATERIALS AND METHODS

### Isolation of the influenza viruses from the clinical samples

The influenza virus A strains used in this study were isolated from the clinical samples of the patients infected by influenza and SARI from different cities of the Russian Federation in the 2014 epidemic season.

The isolation of influenza viruses from the clinical samples was performed in the Madin Darby canine kidney (MDCK) cell line or in chicken embryos according to the Laboratory recommendations for virus isolation [9]. The identification of the isolated influenza strains was performed by means of HAI and polymerase chain reaction (PCR).

### The hemagglutination and hemagglutination inhibition assays

The hemagglutination and HAI assays were performed by using the standard method according to the Practical recommendations [10]. The human erythrocytes (Rh+ group 0) were used in order to enhance the method sensitivity.

### PCR

PCR was accomplished using the reagent set "AmpliSence" ("InterLabService", Moscow) according to the manufacturer's instructions. Isolation of the RNA from the samples was carried out using the "AmpliSenseRIBoprep" and reverse transcription of the virus RNA using the "AmpliSenseReverta" kits. The systems "AmpliSense® Influenzavirus A/B-FL" (analysis of RNA of the influenza A and B virus strains), "AmpliSense® Influenzavirus A type FL" (identification of the subtypes of influenza viruses H1/H3) and "AmpliSense® Influenzavirus A/H1-swine-FL" (detection of the influenza virus A(H1N1)pdm09) were used to perform the real-time PCR.

### Preparation of the mAbs

The mAbs for the influenza A(H3N2) and A(H1N1)pdm09 viruses were prepared according to the described method [11] with some modifications. Balb/c mice or F1 hybrids (Balb/c x SJL/J) were immunized intraperitoneally (ip) by influenza virus purified by ultracentrifugation in the sucrose gradient. In several weeks, animals were boosted by the purified HA of the same virus. In 3 days post last immunization, the splenocytes of immunized mice were hybridized with the cells of the mouse myeloma cell line X63Ag8.653 in the presence of 50% PEG-2000. The cloning of hybrid cells was performed by the limiting

dilution assay. First, the screening of the clones was done by classical ELISA in virus-coated plates with subsequent detection of mAbs by second mouse antibodies bound to horseradish peroxidase (Sigma, USA). Subsequently, cloning of the hybrid clones was done with the use of selective HAT medium. Finally, stable clones were used for the preparation of ascites. For this purpose, Balb/c or F1 hybrid (Balb/c x SJL/J) mice primed by Pristane (0.5 ml per animal) were immunized intraperitoneally by hybrid cells ( $3-5 \times 10^6$  cells per animal). The ascites were collected 2-3 weeks post immunization. All of the work with animals was done in accordance with the "Specific regulations for the use of experimental animals" of the Russian Ministry of Health from 6/19/2003, number 266.

### Micro cell-ELISA for influenza A and B viruses

Virus reproduction in the cells was estimated by the detection of the viral HA in an infected cell. For this purpose, 100  $\mu$ l of the virus containing liquid was added to the 96-well plates (Nunc, Denmark) with a monolayer of MDCK cells. The plates were incubated at 37°C and 5% CO<sub>2</sub>. The medium used for virus cultivation was alpha-MEM ("Biolot", Russia) supplemented with 0.2% of bovine serum albumin (fraction V) (Sigma, USA), 0.05% arginine (Sigma, USA), and 2  $\mu$ g/ml of TPCCK-treated trypsin (Sigma, USA).

After 48-96 h of incubation, whether the cytopathic effect was visible or not, the cell supernatant was removed, the cells were washed with 0.01 M phosphate buffered saline (PBS) pH 7.2, and fixed by 80% cold acetone for 20 min. The cells were washed again with PBS and blocked with 5% skim milk in PBS (PBS-M) for 1 h at 37°C with the subsequent addition of 100  $\mu$ l/well of subtype specific mAb to HA of A(H1N1)pdm09 or A(H3N2) influenza viruses in a concentration of 5-10  $\mu$ g/ml in PBS-M. After a 2 h incubation at 37°C, non-bound mAb were removed and a mouse secondary IgG antibody bound to horseradish peroxidase (Sigma, USA) in PBS-M was added (dilution 1/10,000). The plates were incubated for 1 h at 37°C, washed and the substrate - 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine (TMB) with 0.02% H<sub>2</sub>O<sub>2</sub> in acetate-citric buffer (pH 5.0) was added. The reaction was stopped by the addition of 2 N H<sub>2</sub>SO<sub>4</sub>. The optical density (OD) of the resulting mixture was measured at 450 nm (OD<sub>450</sub>) using spectrophotometer Anthos-2010 (Austria). Samples with uninfected cells were used as a negative control. The results with OD<sub>450</sub> values  $\geq 2OD_{450}$  of the negative control samples were considered positive.

## RESULTS AND DISCUSSION

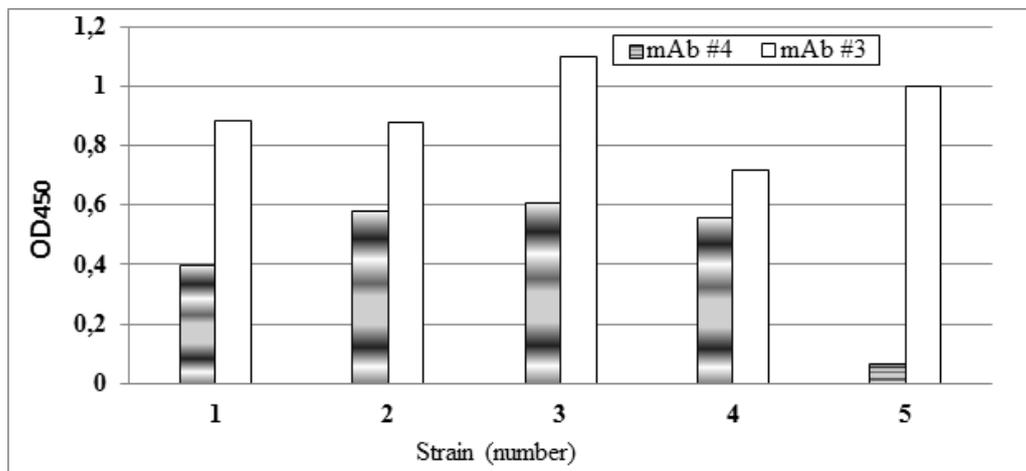
During the epidemic season of 2014, the influenza viruses of A(H1N1)pdm09 subtype were isolated from the clinical samples in chicken embryos (1-3 passages). Viruses of A(H3N2) subtype were isolated in MDCK cell line. The presence of the influenza virus in all the samples was proved by means of the hemagglutination assay with human erythrocytes and by PCR analysis. The hemagglutination titers of isolated viruses varied from 1/4 to 1/64.

Three mAbs: #1 and #2, effectively interacting with the A/Texas/50/2012 (H3N2) and A/Switzerland/9715293/2013 (H3N2) influenza viruses and #4 specifically binding to A/California/07/2009 (H1N1)pdm09 influenza virus, were created. The preliminary screening of these antibodies by immunoblotting, ELISA and HAI assays revealed that every one of them is directed to the HA1 subunit of the corresponding virus and is strongly subtype-specific. In order to confirm virus reproduction in cell lines, the mAb #3 interacting with NP protein of all influenza A viruses was used. Viruses A/California/07/2009 (H1N1), A/Texas/50/2012 (H3N2) and A/Switzerland/9715293/2013 (H3N2) were used as positive controls.

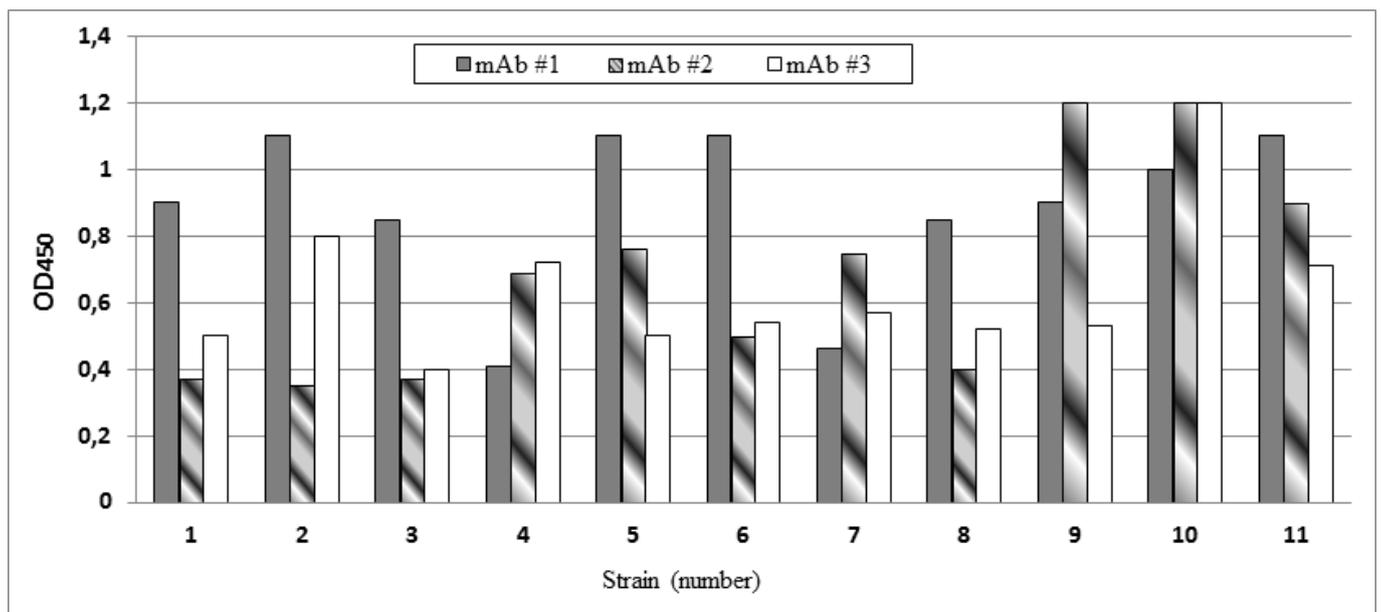
The results presented in Fig.1 demonstrate that all the analyzed viruses reacted with NP specific

mAb #3 while only viruses A/St. Petersburg/1/2014, A/St. Petersburg/16/2014, and A/St. Petersburg/46/2014 reacted with mAb#4 and demonstrated the same level of activity as the control virus A/California/07/2009 of the H1N1 subtype. Virus A/Texas/50/2012 of the (H3N2) subtype did not show any reactivity with mAb #4. These results prove that the viruses A/St. Petersburg/1/2014, A/St. Petersburg/16/2014, and A/St. Petersburg/46/2014 belong to the A(H1N1) subtype.

On the other hand, the viruses A/Astrahan/23/2014, A/Astrahan/24/2014, A/Astrahan/25/2014, A/Astrahan/26/2014, A/Chita/261/2014, A/Chita/258/2014, A/Chita/257/2014, A/St.Petersburg/80/2014, and A/Moscow/252/2014 (Fig.2) reacted with the mAbs #1 and #2 and were similar in this respect to the control viruses



**Fig.1.** Interaction of human influenza viruses with mAbs #3 and #4 in cell-ELISA. Viruses: 1 - A/St. Petersburg/1/2014, 2 - A/St. Petersburg/16/2014, 3 - A/St. Petersburg/46/2014, 4 - A/California/07/2009, 5 - A/Texas/50/2009 (H3N2). The influenza virus A/California/07/2009 (H1N1) was used as the positive control and A/Texas/50/2009 virus of H3N2 subtype as the negative control of the mAb #4. The universal mAb #3 specific to the NP protein of influenza A viruses was used as a control of virus replication.



**Fig.2.** Interaction of human influenza viruses with the monoclonal antibodies #1, #2, and #3 in cell-ELISA. Influenza viruses: 1 - A/Astrahan/23/2014, 2 - A/Astrahan/24/2014, 3 - A/Astrahan/25/2014, 4 - A/Astrahan/26/2014, 5 - A/Chita/261/2014, 6 - A/Chita/258/2014, 7 - A/Chita/257/2014, 8 - A/St. Petersburg/80/2014, 9 - A/Moscow/252/2014, 10 - A/Texas/50/2012, 11 - A/Switzerland/9715293/2013. Influenza viruses A/Texas/50/2009 and A/Switzerland/9715293/2013 of H3N2 subtype were used as the positive control of the mAbs #1 and #2. The universal mAb #3 specific to the NP protein of influenza A viruses was used as a control of virus replication.

A/Texas/50/2012 and A/Switzerland/9715293/2013 of A(H3N2) subtype indicating that all of them belong to the A(H3N2) subtype. Thereby, the subtype differentiation of modern influenza A viruses can be performed by using the cell-ELISA with specific mAbs interacting exclusively with the HAs of the A(H1N1) or A(H3N2) virus subtypes.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## CITATION

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