RESEARCH PAPER

Development of the A/H6N1 influenza vaccine candidate based on A/Leningrad/134/17/57 (H2N2) master donor virus and the genome composition analysis using high resolution melting (HRM)

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ABSTRACT

The avian influenza viruses of H6N1 subtype present a potential danger for humans. The cold-adapted (ca) reassortant influenza virus А/17/herring gull/Sarma/2006/887 (H6N1) was obtained in chicken embryos by the genetic reassortment based on the cold-adapted A/Leningrad/134/17/57 (H2N2) master strain. The genome composition of the obtained reassortant was analyzed by means of real-time PCR with the high resolution melting (HRM) analysis using the intercalating fluorescent dye EvaGreen. Analysis of the gene segments showed that the reassortant A/17/herring gull/Sarma/2006/887 (H6N1) contains the internal proteins coding genes (PB2, PB1, PA, NP, M, and NS) of the master donor virus and the surface antigens coding genes of the A/herring gull/Sarma/51c/2006 (H6N1) avian influenza virus. The study of the phenotypic properties showed that the virus А/17/herring gull/Sarma/2006/887 (H6N1) is temperature sensitive (ts), ca in chicken embryos, and attenuated in mice when administered intranasally. This reassortant can be recommended as a live influenza vaccine candidate for humans.

INTRODUCTION

The influenza viruses of H6N1 subtype that have recently been isolated from wild and domestic birds belong to the family Orthomyxoviridae to the genus Influenzavirus A. Until now, there were no registered severe cases of infection in humans with this type of viruses. However, the serological studies in Southern China revealed that 13% of the inhabitants from different provinces have antibodies to the influenza virus of H6 subtype [1]. Phylogenetic analysis of influenza A viruses indicate that the closely related genes coding the internal proteins could be found in influenza A viruses of different subtypes, and that the reassortment between the avian and human influenza viruses is possible [2]. It was also shown that some of the fragments of the NP and NA genes of highly pathogenic H5N1 viruses originated from the H6 virus of wild ducks [3, 4]. Therefore, the avian influenza viruses of H6N1 subtype present a potential danger for humans. This necessitates the development of a corresponding vaccine strain for the protection of humans from possible infection. One of the candidates for the live influenza vaccine (LIV) – virus A/teal/Hong Kong/97/AA (H6N1) – was obtained on the basis of the A/Ann Arbor/6/60 (H2N2) master strain (donor of attenuation) (Flumist®, USA) using the H6N1 avian influenza virus isolated in 1997 [5]. However, this vaccine virus demonstrated poor reproduction in the respiratory tract of vaccinated patients and low immunogenic activity. The vaccine strains of the live influenza vaccine represent attenuated reassortant virus strains with the genome composition 6:2. The genes coding for the hemagglutinin (HA) and the neuraminidase (NA) are inherited from the modern epidemic virus while the remaining six genes coding the non-glycosylated proteins – from the ca donor of attenuation.

The ca A/Leningrad/134/17/57 (H2N2) (Len/17) donor of attenuation is licensed in the Russian Federation for the production of the LIV vaccine strains for the immunization of adults and children from 3 years old on [6-9]. Previously, we generated and studied the vaccine strains containing the surface antigens of the nonpathogenic avian influenza viruses of A(H5N2), A(H7N3), and A(H9N2) subtypes based on Len/17. The vaccine strains of H5N2 and H7N3 subtypes were tested in clinical trials and showed high immunogenic activity inducing the seroconversions in 54.8% and 72% of the patients, respectively after double immunization. The goal of the present research project was to obtain the vaccine candidate of H6N1 subtype based on the master strain Len/17 with the surface antigens of the influenza virus A/herring gull/Sarma/51c/2006 (H6N1) and to study its properties in vitro and in the laboratory animals.
The traditional analysis of the genome composition of the vaccine candidates obtained by the classical genetic reassortment is performed by the specific restriction of the gene products amplified by polymerase chain reaction (PCR) [10] or by the multiplex PCR [11] with the complete sequencing of the reassortant’s genes. For the fast genome composition analysis of the potential vaccine candidates, the real-time PCR with the use of the hydrolysable oligonucleotide probes [12] and pyrosequencing is used [13]. In order to simplify the procedure for the vaccine candidates screening we suggest using PCR combined with the high-resolution melting curves analysis (HRM-analysis). This method saves time compared to the visualization of results by electrophoresis in the course of PCR analysis and lowers the risk of contamination with amplicons. Furthermore, this method is relatively cheap because it does not require the use of expensive fluorescent oligonucleotide probes.

### MATERIALS AND METHODS

#### Viruses

The following influenza viruses were used in this research project: the donor of attenuation Len/17 and the wild type (wt) pandemic virus A/Leningrad/154/57 (H2N2) (Len/wt) received from the Department of Virology of the Institute of Experimental Medicine, as well as the avian influenza virus A/herring gull/Sarma/51s/2006 (H6N1) (H6N1/wt) isolated by Dr. A. Lyapunov from Irkutsk State University. The viruses were cultured in 10-day-old chicken embryos (Skovorcy, Russia). The virus infectious activity in the chicken embryos was determined at the optimal (33°C), elevated (up to 39°C), and lowered (up to 26°C) temperatures. The 50% embryonated infectious dose EID₅₀ was calculated with the use of the Reed – Muench method [14].

#### PCR with HRM-analysis

The virus RNA was isolated from 80 μl of the virus containing allantoic fluid by means of the QIAamp Viral RNA Mini Kit (Qiagen, the Netherlands). The obtained RNA samples were stored at -20°C. Reverse transcription (RT) was performed using the reverse transcriptase of the leukemia Molony mouse virus (M-MulV, SibEnzyme) and random hexamers. The RT- PCR was conducted using the thermal cycler CFX96 (Bio-Rad, USA) with the use of expensive fluorescent oligonucleotide probes. This method saves time compared to the visualization of results by electrophoresis in the course of PCR analysis and lowers the risk of contamination with amplicons. Furthermore, this method is relatively cheap because it does not require the use of expensive fluorescent oligonucleotide probes.

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The reaction was performed in 20 μl containing 10 μl of reaction mixture SscqFast™ EvaGreen® Supermix (Bio-Rad, USA), 0.5 μMol of each primer, 4 μl of cDNA and pure water (RNase-free) according to the manufacturers’ recommendations. Melting of the PCR products was accomplished at temperatures from 65°C to 95°C with 0.5°C increments. HRM-analysis of the melting curves was performed with the help of Precision Melt Analysis Software, Version 1.1 (Bio-Rad, USA).

#### Sequencing according to the Sanger method

In order to perform the partial sequencing the DNA-copies of the PB2, PB1, PA, NP, and NA RNA segments were obtained using the OneStep RT-PCR Kit (Qiagen, the Netherlands). Following the electrophoresis of the DNA-copies in 1.5% agarose gel and consecutive purification by QIAquick PCR purification Kit (Qiagen, the Netherlands), the sequencing was conducted on DNA-analyzer ABI 3730xl using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA). The processing of the nucleotide sequence data was performed using the 3750 Data Collection v3.0 software package (Applied Biosystems, USA). The partial nucleotide sequences of PB2, PB1 and NP genes were deposited to the GISAID EpiFlu database with the corresponding access numbers PB2-EPI774106, PB1-EPI774107, and NP-EPI774105.

#### The determination of the origin of the surface antigens coding genes

The HA origin was proved by hemagglutination inhibition assay (HAI) with the specific rat antisera. The origin of the NA gene was determined by partial sequencing of the gene segments obtained by RT-PCR using primers NA-F1 01 (5’ GATTAGCTCAACCCAGAAAC 3’) and NA-R605 (5’ A GAAAAAGAAAAAGTAAATAC 3’).

#### The investigation of the reassortant properties following the intranasal immunization of mice

The 10-week-old CBA mice (Rappolovo, Russia) were immunized intranasally under the mild ether anesthesia with 50 μl of virus containing allantoic fluid with 10⁶ EID₅₀ of virus. The sterile phosphate buffered saline (PBS) was used as a placebo. Animal euthanasia was performed according to the “Regulations for conducting of experiments with the use of animals”. The virus load in the mouse lungs and nasal turbinates (4 animals per group) was determined on the third day post immunization by titration of the corresponding organ’s suspension in the chicken embryos starting from the 1:10 dilution for the lungs and from 1:2 dilution for the nasal turbinates. The blood samples were collected three weeks post immunization. The obtained sera (7 animals per group) were treated with the neuraminidase inhibitor (Denka-Seiken, Japan) and tested for the presence of antibodies to the vaccine virus Len17/H6 in HAI test as described by Rowe et al. [16].

#### Statistical analysis

The experimental data were processed using the statistical software package ‘Statistika’ (version 6). The comparison of the two independent groups was conducted using the nonparametric Mann-Whitney criterion. The difference was considered to be statistically significant at p < 0.05.
RESULTS AND DISCUSSION

The reassortant vaccine candidate Len17/H6 was obtained by the classical reassortment of the avian influenza virus H6N1/wt and ca donor of attenuation Len/17 as described by Alexandrova [17]. The origin of the HA gene of the Len17/H6 from the avian influenza virus H6N1/wt was confirmed by HAI with the specific rat antisera. The reassortant’s NA gene is also originated from the H6N1/wt virus as revealed by sequencing the NA gene segment.

The origin of the rest genes was determined by the RT-PCR following by HRM-analysis using the intercalating fluorescent dye EvaGreen. The RT-PCR with the use of the nonspecific DNA-binding dyes usually includes the analysis of the PCR products melting curves. For that, the temperature in the probes is gradually enhanced which leads to the fluorophore release from the denatured double-stranded DNA. The rate of the fluorescence change allows for determining the peak corresponding to the melting point of the double-stranded DNA complexes. The dimers of primers usually have a lower melting point due to their smaller size that allows distinguishing the amplified PCR fragments from dimers of primers and other nonspecific products.

The analysis of the high resolution melting curves suggested by Wittwer et al. [18] generates melting curves profiles that are specific and sensitive enough for distinguishing DNA with slight differences in sequence that makes it possible to scan the mutations, analyze methylation, and perform genotyping [19]. HRM-analysis can be used for the characterization of the samples based on their CG-composition and complementarity of the DNA sequences. The growing tendency to simplify the mononucleotide polymorphism analysis leads to the rapid development of HRM-technology. In order to perform the high resolution melting curves analysis the use of the third generation of the intercalating dyes such as EvaGreen, LCGreen and SYTO9 is recommended [20]. These dyes have low toxicity; they do not inhibit the PCR process and could be used in RT-PCR at higher concentrations than SyberGreen. This enables reaching a higher saturation of the double-stranded DNA with the dye and reduces the redistribution of the dye to the non-denatured areas of DNA in the course of melting.

HRM-analysis is used already for the detection and quantification of influenza viruses [21] as well as for the screening of the new emerging strains in the population [22].

The results of HRM-analysis of the Len17/H6 reassortant internal and nonstructural proteins are shown in Fig. 1. The quantitative clusterization analysis for all positive wells was performed using the software Precision Melt Analysis. For the visual clusters identification, this software generates so-called ‘differentiation curves’ for every well, which shows the difference in fluorescence.

Fig. 1. HRM-analysis of the genes of the inner and nonstructural proteins for the vaccine candidate Len17/H6 and the parent viruses. The cumulative data from the two experiments are presented.
Fig. 2. The analysis of the Len17/H6 genome composition by RT-PCR with subsequent restriction analysis. The gene fragments of PB2 (1374-1614), PB1 (740-1044), PA (900-1077), NP (886-1200), M (59-249), and NS (673-830), of the vaccine candidate Len17/H6 and the parent viruses were amplified by RT-PCR. The obtained PCR products of PB2, NP, M and NS genes were treated with the restriction endonucleases Tru 9I, Eco RI, Bst FNI and Cac8I, respectively, and analyzed by electrophoresis in the agarose gel. M – DNA marker, N – negative control; “+” - the fragment was treated with the corresponding restriction enzyme, “-” – no treatment with the restriction enzyme.

Fig. 3. HRM-analysis of the PB1 and PA genes of the vaccine candidate Len17/H6, parent viruses and the pandemic virus Len/wt with the subsequent proof of the amplified fragments by sequencing. The genes fragments of PB1 (781-952) and PA (886-1200) of the vaccine candidate Len17/H6, parent viruses and the pandemic virus Len/wt were amplified and the corresponding high resolution melting curves were compared. The amplified fragments were also sequenced and the resulting alignment is presented.
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between the well and the reference curve. The latter is defined as the average among all the fluorescent curves for the corresponding reference cluster. It is quite clear from the graph that the shapes of the melting curves and the temperature profile for the virus H6N1/wt and for the donor of attenuation Len/17 are different. Based on that, it is possible to conclude that the genes coding the inner and nonstructural proteins of the reassortant Len17/H6 are inherited from the ca donor of attenuation Len/17.

The verification of obtained data was performed by classical RT-PCR with the subsequent specific restriction analysis according to method [10]. As it is shown in Fig. 2, the restriction enzymes cut the DNA fragments of the PB2, NP, M, and NS genes of the master strain and the vaccine candidate that indicates the presence of specific nucleotides typical for the donor of attenuation (PB2 and NS) or for the H2N2 viruses (NP and M). At the same time, the amplified fragments of avian influenza viruses are not cleaved at the same conditions. These findings confirm the results obtained with HRM-analysis and prove that the melting of the PCR products does not destroy the DNA duplex structure, which enables the analysis of the amplified fragments by other methods. However, as shown in Fig. 2, there is no amplification of the PB1 and PA genes fragments of H6N1/wt. In this connection, new primers for the PB1 and PA genes were developed in order to amplify the shorter fragments (781-952 and 47-206, respectively). The origin of the PB1 and PA genes fragments amplified with the new primers was confirmed by the sequencing and HRM-analysis (Fig. 3). The data presented in Fig. 3 also show a clear difference between the melting curves for the gene fragments of the master strain Len/17 and Len/wt virus, which differ only by one nucleotide substitution.

Thereby it is proved that the reassortant Len17/H6 acquired six genes coding the inner and nonstructural proteins from the donor Len/17. The search for the homologous sequences in GenBank performed with the BLAST [23] showed that the PB1 gene of H6N1/wt virus is the most similar (99% of homology) to the avian virus A/duck/jiangxi/5945/2008 (H6N1) (GenBank KP287703.1), whereas the closest PA gene belongs to the avian virus A/duck/Guizhou/2492/2007 (H6N1) (GenBank, CY109657.1). The comparison of the amplified PB1 gene sequences of the Len/17 and Len17/H6 with the H6N1/wt revealed many differences. One of them is the substitution T819→A that is the part of the specific cleavage site and enables to distinguish these genes by specific restriction analysis (Fig. 5). The PA gene sequences of the reassortant and the donor of attenuation are also different in many positions when compared to the H6N1/wt, and the avian virus A/duck/Guizhou/2492/2007 (H6N1), but all these viruses contain the nucleotide C at position 107. Since this nucleotide is critical for the traditional restriction analysis it is problematic to distinguish these genes by this method. These results confirm the limited possibilities of the commonly used restriction analysis in the course of genotyping of the avian influenza virus vaccine strains made on the base of Len/17 and prove the necessity of new methods for the vaccine candidates screening.

In the course of the cultivation of the Len17/H6 in chicken embryos at different temperatures it was shown that this virus is ts and ca as well as the master strain Len/17 in contrast to the H6N1/wt strain. The difference in titers at optimal (33°C) and elevated (39°C) temperatures reached 7.0 log<sub>10</sub> EID<sub>50</sub>/ml. The difference at the reduced and the optimal temperatures did not exceed 3.0 log<sub>10</sub> EID<sub>50</sub>/ml (Table 1).

Upon the intranasal immunization of mice with the dose of 10<sup>6</sup> EID<sub>50</sub> the virus Len17/H6 grew in the animals lungs until the titer 2.8 log<sub>10</sub> EID<sub>50</sub>/ml that was below the titer of the parent H6N1/wt virus and corresponded to the level of reproduction of the ca master strain Len/17 (Table 1). At the same time, the vaccine strain Len17/H6 grew effectively in the mouse nasal turbinates, where the temperature is significantly lower. Moreover, the virus Len17/H6 caused the systemic immune response in mice three weeks post the single immunization with the antibody geometric mean titer (GMT) of 17.4. It should be mentioned that the vaccine candidate Len17/H6 demonstrated the highest immunogenicity in mice after the single immunization compared to other vaccine candidates obtained with the apathogenic avian influenza viruses. Therefore, the antibody GMT obtained in mice after the single immunization with potentially pandemic H5N2 vaccine candidate was 6.6 (p=0.047), H7N5 – 8.7 (p=0.047) and H9N2 – 5.0 (p=0.0009).

Thereby, the results of the present research showed that the HRM-analysis enables performing the vaccine candidates screening. Specific restriction analysis method and partial sequencing confirmed the genome composition of the reassortant Len17/H6, which was determined by HRM-analysis. Analysis of the Len17/H6 phenotypic properties in vitro (ts and ca phenotypes) and in vivo (attenuation for mice and the immune response after the single intranasal immunization) leads to the

Table 1. The comparison of the biological properties of the reassortant vaccine candidate Len17/H6 and the parent strains

| Virus                     | Reproduction in the chicken embryos, log<sub>10</sub> EID<sub>50</sub>/ml | Virus titer in the mouse respiratory tract on day 3, log<sub>10</sub> EID<sub>50</sub>/ml | GMT of antibodies |
|---------------------------|-----------------------------|---------------------------------|-------------------|
|                           | 26°C                        | 33°C                           | 59°C              | Lungs      | Nasal turbinates |                              |
| Vaccine candidate         | Len17/H6                    | 6.5±0.8                        | 9.3±0.3           | 2.3±0.3    | 2.8±0.8        | 3.3±0.5                       | 17.4                          |
| Donor of attenuation      | Len/17                      | 7.9±0.7                        | 9.3±0.5           | 2.2±0.6    | 2.3±0.7        | 2.5±0.9                       | ≤10                           |
| Wild type virus           | H6N1/wt                     | 1.5±0.0                        | 6.7±0.1           | 7.2±0.4    | 4.1±0.3        | 2.8±0.9                       | 16.8                          |
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conclusion that the vaccine candidate Len17/H6 meets the requirements for the LIV vaccine candidates.

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CONFLICT OF INTEREST STATEMENT

The authors declare no commercial or financial conflict of interest.

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