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Original Article

Development and Sequence Analysis of a Cold-Adapted Strain of Influenza A/New Caledonia/20/1999(H1N1) Virus

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Abstract

Background and Aims: Vaccination is the most effective method to prevent influenza infection. Among the available vaccines, cold-adapted live-virus vaccines are suitable approach that have been produced and evaluated for recent years in few countries. The goal of this project was to derivate a cold adapted variant of the influenza A/New Caledonia/20/1999(H1N1).

Materials and Methods: Influenza A/New Caledonia/20/1999(H1N1) was adapted to grow at 25°C by gradually decreasing the incubation temperature through the sequential passages in embryonic eggs. The viral genome extracted from the starting seed and the last round of passage at 25°C was amplified by RT-PCR. The amplified cDNA fragments were subjected to sequencing determination bi-directionally. Sequence data were aligned to find mutated positions.

Results: Sequence analysis showed totally six cases of point mutations that five of them resulted in amino acid substitutions and one of them was a silent mutation. These substitutions of one amino acid occurred in PB2, PA, NP proteins and two amino acid changes in HA protein sequence.

Conclusion: The variant of cold adapted strain made here could be used as a master donor to generate attenuated reassortant influenza vaccine viruses.

Keywords: Influenza Viruses Type A; cold adapted; vaccine; analysis

Introduction

Influenza A viruses, as an important pathogens affecting human population, are segmented, single-stranded, and negative-sense RNA viruses and a member of Orthomyxoviridae. The eight segments encoding at least 11 known proteins (1). Two of them are surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA) which are embedded in the envelope. The classification of Influenza A viruses is based on the antigenic properties of these glycoproteins. So far, 16 subtypes of HA and 9 subtypes of NA have been identified (2).

Antigenic variability of the virus is responsible for annual epidemics and occasional pandemics of the disease in human. Every year, researchers and manufacturers reexamine available vaccines and develop a new one for prophylaxis of influenza virus that is a reoccurring infection in human. One of the major class of viral vaccines is live attenuated vaccine. The only FDA-licensed live product against influenza is cold-adapted(ca), live attenuated Influenza Vaccine (LAIV) (3). Such reassortant viruses are immunogenic, satisfactorily attenuated and phenotypically stable (3). The first step in developing LAIV is

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to obtain of a cold-adapted and stably attenuated master donor virus. Cold-adapted influenza virus strains were originally produced through adaptation of the virus to grow at 25°C by sequential passages of the wild type virus in embryonic eggs at progressively lower temperature (4, 5).

Two cold adapted influenza virus variants, A/Leningrad/134/17/57(H2N2) and A/Leningrad/134/47/57(H2N2) strains, have been used as a donor of attenuating genes for the construction of live-attenuated reassortant influenza vaccine viruses in Russia (6, 7). Another cold adapted influenza virus, A/Ann Arbor/6/60 (H2N2), has been available as a master donor for many years in the USA (4, 8). These vaccines that are delivered by the mucosal route are attenuated, genetically stable, immunogenic and specially induce mucosal antibody.

The aim of this study was to produce a new ca influenza virus variant and to determine of its complete genome sequence in order to obtain information on the molecular basis of the attenuation.

Methods

Virus

H1N1 (A/New Caledonia/20/1999(H1N1) was obtained from the National Influenza Centre, Tehran Medical Science University.

Chicken embryo infection

For each passage, appropriate dilution of virus was prepared in PBS and 0.1 ml of each dilution was injected into the allantoic cavities of 11-day-old chicken embryo. The incubation temperature at started from 37°C and followed by 3 passages at 36°C, 5 passages at 35°C, 5 passage at 27°C, 7 passage at 28°C , 8 passage at 27°C, 15 passage at 26°C and 23 passage at 25°C. The infected eggs were incubated for 3 days at above mentioned temperatures and then were placed at 4°C overnight. The Hemagglutination Assay (HA) was used to screen viral particles in allantoic fluid harvested from the embryonic chicken eggs. The allantoic fluids were diluted in PBS from 1:4 to 1:1024 in U-shaped 96-well plate. 0.5% suspension of chicken red blood cells was added to each well. Plates were incubated for 30 min at room temperature and hemagglutination was assessed by eye.

RNA isolation and RT-PCR

RNA was isolated from 150 µl of allantoic fluid from the 23th passage at 25°C (the last round of sequential passages) using RNX™-PlusKit (CinnaGen, Iran) according to the manufacturer instructions. The extracted RNA was eluted into 50 µl of DEPCE treated water. One step RT-PCR was employed to amplify each of the viral gene segments using one step RT-PCR kit (Qiagen- Cat no. 210212). The RT-PCR reactions were done in volume of 50 µl, according to the instruction of the manufacturer. The oligonucleotide primers used in this study, were introduced previously by Hoffmann *et al* (9). The Amplification was performed with the following parameters: 1 cycle at 50°C for 30 min and 95°C for 15 min, followed by 45 cycles at 95°C for 0.5-1 min, 60°C for 20s, and 72°C for 30s, with a final elongation step at 72°C for 10 min. Apart from the annealing temperature, the termoprofile for amplification of all regions was the same. The annealing temperature was considered 58°C for PB2, PB1, PA and NP segments and 56°C for HA, NA, M and NS segments. The PCR products were visualized on 1% ethidium bromide stained agarose gel.

Sequence Analysis

PCR products were subjected to bi-directional sequencing using specific primers for each gene to determine the probable mutations throughout the whole genome. The nucleotide sequence of all eight segments and related deduced amino acid sequence of ca influenza virus were aligned with reference sequence of influenza virus H1N1 (A/New Caledonia/20/1999(H1N1) (accession number DQ508854- DQ508861). Control of sequencing results, editing of raw data and alignment analysis were accomplished by the Choromas (version 1.45-Australia) and Bioedit (version 5.0.9Tom Hall, Department of Microbiology, North Carolina State University) softwares.

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Fig. 1. Agarose gel electrophoresis of RT-PCR products carried on RNA extracted from propagated viruses of the 23th passage at 25°C (the last round of sequential passages). Lane 1, PB2 (bp 1536); Lane 2, PB1 (1213 bp); Lane 3, PA (1402 bp); Lane 4, HA (721 bp); Lane 5, size marker 1Kb Fermantas; Lane 6, NP (1191 bp); Lane 7, NA (836 bp); lane 8, M (510 bp); Lane 9, NS (635bp).

Results

Influenza virus H1N1 (A/New Caledonia/20/1999(H1N1) were cultured in embryonic eggs. The incubating temperature was reduced gradually so that the temperature reached 25°C. We often observed that after each step of decreasing temperature, the viral titer also decreased. In such cases, by repeating the cultivation at the same temperature as well as increasing the number of inoculated eggs, the viral titer was increased.

To identify gene mutation of the cold adapted variant, the whole genome of the isolate from the last round of passage at 25°C were amplified by RT- PCR. Figure1 shows the amplification result of eight genomic segments. Then PCR products were subjected to bi-directional sequencing. Data analysis indicated that the six point mutations occurred through the complete genome of the ca virus in comparison with the reference (starting strain) sequence. Two of them were identified in the PB2, one in the PA, one in the NP and two in the HA genes. Comparison of the deduced amino acid sequences for the parental and ca virus revealed totally four mutations resulted in amino acid substitutions in PB2, PA, NP and HA proteins (Table 1).

Discussion

Influenza remains a serious disease that causes numerous deaths every year around the world. Primary prevention of influenza infection is based on vaccination. Currently, vaccines that are used in Iran and most of the country are mainly inactivated vaccines. However, the production and application of inactivated vaccine is associated with some problems and also their efficacy is suboptimal in the children and the elderly (10-12). Another approach for prevention of influenza infection is LAIV. This type of vaccine has been investigated and produced as monovalent , bivalent and trivalent in the United States since 1960 (13). These vaccines are not toxic and moreover they are genetically stable and will not be converted to wild-type after administration. However, cold-adapted live-virus vaccines are the only FDA-licensed product against influenza.

The virus strain of such vaccines is produced by genetic reassortant between the ca mutant and the strain of viruses involved in the epidemic. The reassortant strain contains HA and NA antigens of circulating virus and internal genes of the ca mutant.

In the present study, influenza virus H1N1 (A / New Caledonia/20/1999 (H1N1) strain was adapted to grow at 25°C by gradually lowering the incubation temperature in embryonic eggs. Then the genome of ca strain was compared with its parental wild-type strain at nucleotide and amino acid level.

We have identified six nucleotide differences between the A/New Caledonia/20/1999(H1N1) ca mutant strain and its wild type parent. Alignment of deduced amino acid sequences of the parental and mutant viruses revealed five amino acid substitutions. Mutations were found in the genes coding for PB2, PA, NP and HA proteins. Among the influenza proteins, HA is more variable cause in a non-favorable

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Table 1. Nucleotide and amino acid changes of the ca strain relative to wt H1N1 Caledonia/20/1999(H1N1) virus.

Gene segment	nucleotide changes	Amino acid changes
PB2	G807A,G1305A	A270T
PB1	—	—
PA	G1027A	E343K
HA	G831A, G431A	T144N, G278R
NP	G838A	V280M
NA	—	—
M	—	—
NS	—	—

condition of proliferation, mutations will be occurred through it more faster than the other proteins (14). Therefore, the observed mutation in this segment may be related to number of passages rather than cold adaptation. Several studies investigated the possible roles of NP protein in transcription, replication, intracellular trafficking and accumulation of the viral genome in the infected cells [15, 16]. Probably mutations that occurred in this protein prevent replication, transportation, and accumulation of the virus. A/Leningrad/134/17/57 (H2N2) and A/Ann Arbor/6/60 are ca mutants that are used as a Master Donor Viruses (MDVs) in reassortment with current wild type influenza A, to generate attenuated vaccine viruses containing the proper surface glycoproteins (17, 18). Both of these MDVs have been studied at the gene level. It is reported that PB2, PB1 and NP genes of A/Ann Arbor/6/60 and PB2, PB1 and PA gene segments of A/Leningrad/134/17/57 play an essential role in temperature sensitivity and attenuation of these variants (19, 20). Moreover, B/USSR/60/69 is another strain of ca that is used as MDV for preparing reassortant influenza B trivalent live attenuated influenza vaccine in the Russian. It is demonstrated that mutations in the polymerase genes of PB2 and PA play an essential role in cold attenuation of B/USSR/60/69 MDV (21) although the molecular mechanism of cold adaptation is not recognized well. Nevertheless, Based on our results and the data published previously, the sequential passage of virus in embryonic egg in low temperature condition, caused selection of those variants containing the mutations that is beneficial for the growth of these viruses. Meanwhile, these variant could not grow at higher temperature. The results have shown that these type of variants are genetically stable and reversion of the genes carrying temperature-sensitive mutations to wild type is rare (5). Stability of these variants can be explained in this manner that probably both of low temperature and arisen mutations in polymerase gene can reduce the rate of viral replication (22) and in this condition viral polymerase makes less mistakes.

The variant of ca A/New Caledonia/20/1999(H1N1) mutant virus developed here may potentially exploit as a master donor to create a live attenuated vaccine through reassortment with a wild type influenza virus isolate.

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