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Genotyping and screening of reassortant live-attenuated influenza B vaccine strain

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Live-attenuated influenza virus vaccines can be generated by reassortment of gene segments between an attenuated donor strain and a virulent wild-type virus. The annual production schedule for the seasonal influenza vaccine necessitates rapid and efficient genotyping of the reassorted progeny to identify the desired vaccine strains. This study describes a multiplex RT-PCR system capable of identifying each gene segment from the cold-adapted attenuated donor virus, B/Lee/40ca. The specificity of the amplification system was optimized by testing various wild-type influenza B viruses. The resulting RT-PCR method is sensitive and efficient enough for routine identification of reassortant clones to identify the desired gene constellation, consisting of six segments from the attenuated donor virus and the H and N genes from the wild-type virus. By providing a more rapid and efficient means of genotyping the candidate reassortant strains, this method could be implemented to expedite the generation of each component strain and allow more time to culture and process the final seasonal influenza vaccine.

1. Introduction

Influenza virus is an enveloped virus with a negative-sense segmented RNA genome that belongs to the *Orthomyxoviridae* family (Beigel, 2008). Influenza A and influenza B viruses are globally important respiratory human pathogens that cause annual epidemics and occasional pandemics necessitating annual vaccination against newly circulating strains. Annual influenza epidemics are caused typically by antigenic drift, point mutations within gene segments, while pandemics are typically caused by antigenic shift, the reassortment of a whole gene segment that changes completely the antigenicity of a circulating virus (Carrat and Flahault, 2007; Hampson and Mackenzie, 2006; Wright et al., 2007). Influenza specific antiviral drugs are able to reduce the extent of virus spread throughout the population (Beigel and Bray, 2008; De Clercq, 2004; Intharathep et al., 2008; Moscona, 2008), but vaccination remains the primary means of protecting people against influenza disease.

Every year the World Health Organization (WHO) predicts the virus strains likely to circulate in the upcoming influenza season in each hemisphere and recommends three strains, two influenza A types and one influenza B type, to be included into the vaccine formulation. Currently, inactivated influenza vaccines are licensed worldwide, but live-attenuated influenza vaccines are used only in Russia and have been licensed recently in the USA (Bardiya and Bae, 2005; Belshe, 2004; Ohmit et al., 2008). Immunization with the live-attenuated influenza vaccine induces a broader immune response that resembles natural infection, which establishes better long-term immunity against influenza virus (Cox et al., 2004; Wareing and Tannock, 2001).

The segmented nature of the influenza genome allows attenuated reassortant viruses to be produced by co-infecting cells with an attenuated influenza strain, which serves as the source of attenuated genes and a circulating wild-type human influenza virus. The co-infection results in a pool of reassortant viruses with a random combination of the eight RNA genomes from the two parental viruses. Among the 256 possible reassortants, it is necessary to isolate and identify the reassortant that inherited the two gene segments encoding the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins from the epidemic wild-type virus and the remaining six gene segments (PB2, PB1, PA, NP, M, and NS) from the attenuated donor virus (Maassab and Bryant, 1999; Seo et al., 2008). The tight schedule associated with the annual production of the trivalent influenza vaccine necessitates an accurate and rapid method to genotype reassortants to identify clones with the desired genetic constellation. Recently a multiplex RT-PCR

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technique was developed to identify rapidly gene segments originating from a live-attenuated influenza A virus strain (Ha et al., 2006). This report presents a similar multiplex RT-PCR system for genotyping reassortant influenza B viruses. This method could be implemented to facilitate progeny genotyping and expedite the production of the trivalent live-attenuated influenza vaccines each year.

2. Materials and methods

2.1. Viruses and cell

The cold-adapted influenza B virus (B/Lee/40ca) was generated from repeated passages at low temperature in embryonated hen's eggs, as previously described (Seo et al., 2008). Wild-type influenza B viruses (B/Shangdong/7/97, B/Hong Kong/330/2001, B/Jillin/20/2003, B/Shanghai/361/2002, B/Yamagata/16/88, B/Beijing/76/98, B/Panama/45/90, B/Hawaii/10/2001, and B/Jiangsu/10/2003) were obtained from the National Institute for Biological Standards and Control (NIBSC, UK). All viruses were propagated in 11-day-old specific-pathogen-free (SPF) embryonated hen's eggs (Charles River, Jinan, China) or in Madin–Darby canine kidney (MDCK) cells, which were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum. Virus-infected MDCK cells were maintained in MEM with 0.025 μg/ml of TPCK-treated trypsin (Invitrogen, Life Technologies, Carlsbad, CA, USA).

2.2. Primer selection

Primers were designed on the basis of sequence information from the Influenza Sequence Database of Los Alamos National Laboratories, Los Alamos, New Mexico (http://www.flu.lanl.gov). Multiple sequence alignments were performed using the alignment program of MAFFT version 6 from the Medical Institute of Bioregulation, Kyushu University (http://align.bmr.kyushu-u.ac.jp/mafft/online/serv-er). Regions of naturally occurring sequence diversity were identified to design virus strain-specific primers. The primers were designed to have an annealing temperature of ~58–60 °C and to minimize base-paring at the 3′-end of the primer binding site for the majority of wild-type influenza B viruses. Positive control primers were chosen to amplify the two internal PB2 and PB1 genes that are highly conserved among all influenza B viruses (PB2-F: 5′-gcaggaataccaagagaatc-3′; PB2-R: 5′-tcttgagaaaataccatgca-3′; PB1-F: 5′-tagtagttgaaaacttccc-3′; and PB1-R: 5′-cagtaactttttcttttgctc-3′). All of the primers were synthesized using a DNA synthesizer (PerkinElmer, Foster City, CA, USA and Cosmogenetech, Seoul, Korea).

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from virus-infected MDCK cells grown in 6-well plates (about 1×10^5 cells per well) using the easy-BLUE™ Total RNA Extraction kit (INtRON Biotechnology, Korea) according to the manufacturer's protocol. The concentration of RNA was measured spectrophotometrically at 260 nm, and stored at −70 °C. The reverse transcription (RT) reaction was performed using the Omniscript Reverse Transcriptase kit (Qiagen, Valencia, Germany) according to the manufacturer's protocol. Each reaction contained 5.25 μl RNase-free water, 1 μl 10× RT buffer, 1 μl 5 mM of each dNTPs, 0.5 μl Omniscript reverse transcriptase, 0.25 μl RNase inhibitor (10,000 unit), 10 pmol synthesized 18mer oligo-dT primer, and 800 ng total RNA (1 μl). The thermal cycler program for the RT reaction was as follows (per cycle): 37 °C for 60 min, 95 °C for 5 min, and 4 °C for 5 min.

2.4. Polymerase chain reaction (PCR)

The PCR was performed using 50 ng cDNA and 48 μl of a master mixture containing 28.5 μl distilled water, 5 μl 1× i-Taq enzyme buffer (INtRON Biotechnology, Korea), 4 μl 4 mM of each dNTP (Takara, Japan), 10 μl of a 10 pmol mixture of each primer set, and 1 U i-Taq polymerase (0.5 μl) (INtRON Biotechnology, Korea). The reaction was performed on a PE 9700 thermocycler (Applied Biosystems, PerkinElmer) using the following cycling conditions: 94 °C for 5 min, 25 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 2 min, and a final extension step at 72 °C for 7 min. The PCR products were resolved by electrophoresis on 2% agarose gels, stained by ethidium bromide (0.2 μg/ml) and visualized by a UV transilluminator.

2.5. Sequencing

Amplified RT-PCR products from the reassortant viruses were sequenced directly to confirm their origins. Sequencing was performed by Cosmogenetech, Seoul, Korea, using the ABI 3730XL automated sequencing machine (Applied Biosystems, Foster City, CA, USA).

3. Results

3.1. Design of B/Lee/40ca specific primers

Strain-specific and segment-specific primer pairs were designed to match the B/Lee/40ca genome but result in mismatched base pairs at the 3′-end of the primer binding site for non-homologous wild-type influenza B viruses. The number of individual influenza B virus sequences used for the alignment of each gene segment were 56 for PB2, 65 for PB1, 64 for PA, 59 for HA, 70 for NP, 110 for NA, 58 for M, and 50 for NS gene. Each primer pair was validated to amplify specific products from the attenuated vaccine donor strain B/Lee/40ca and not to amplify products from wild-type influenza B viruses. To determine the specificity of the primer pairs, five influenza B strains (B/Lee/40ca, B/Panama/45/90, B/Beijing/76/98, B/Jilin/20/2003, and B/Shangdong/7/97) were examined. From the initial screening, low-specificity primer pairs were excluded and primer pairs with high-specificity (Table 1) were further tested using five additional wild-type influenza B virus strains (B/Yamagata/16/88, B/Shanghai/361/2002, B/Jiangsu/10/2003, B/Hawaii/10/2001, and B/Hong Kong/330/2001) (data not shown). A subset of the validation experiments are shown in Fig. 1, and a summary of the amplified PCR products for each virus strain/primer set combination are shown in Table 2. The positive control primers were chosen from conserved regions of the PB2 and PB1 genes and included in each reaction (Fig. 1, lanes 19, 20, 36, and 37).

3.2. Selection of multiplex primer sets specific for the cold-adapted donor virus

A multiplex RT-PCR format was developed that enabled the simultaneous amplification of multiple RNA influenza gene segments. Since the RT-PCR products for the different gene segments can be easily distinguished by size, three different multiplex reactions were tested, each composed of two or three segment-specific primer pairs (Table 3). All eight segment-specific PCR products were amplified successfully when B/Lee/40ca RNA was used as a template and no nonspecific bands were observed. Further, no PCR