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Titration of individual strains in trivalent live-attenuated influenza vaccine without neutralization

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A B S T R A C T

Formulation and quality control of trivalent live-attenuated influenza vaccine requires titration of infectivity of individual strains in the trivalent mix. This is usually performed by selective neutralization of two of the three strains and titration of the un-neutralized strain in cell culture or embryonated eggs. This procedure requires standard sera with high neutralizing titer against each of the three strains. Obtaining standard sera, which can specifically neutralize only the corresponding strain of influenza viruses and is able to completely neutralize high concentration of virus in the vaccine samples, can be a problem for many vaccine manufacturers as vaccine stocks usually have very high viral titers and complete neutralization may not be obtained. Here an alternative approach for titration of individual strain in trivalent vaccine without the selective neutralization is presented. This was done by detecting individual strains with specific antibodies in an end-point titration of a trivalent vaccine in cell culture. Similar titers were observed in monovalent and trivalent vaccines for influenza A H3N2 and influenza B strains, whereas the influenza A H1N1 strain did not grow well in cell culture. Viral interference among the vaccine strains was not observed. Therefore, providing that vaccine strains grow well in cell culture, this assay can reliably determine the potency of individual strains in trivalent live-attenuated influenza vaccines.

1. Introduction

Live-attenuated influenza vaccine (LAIV) is being widely used in many countries (Wareing and Tannock, 2001; Jin and Subbarao, 2015). The manufacturing process is less complicated and the yield per embryonated egg is much higher than inactivated vaccines (Rudenko and Isakova-Sivak, 2015). This is an attractive option for vaccine production in a pandemic, when the number of embryonated eggs is the main limiting factor for total production capacity (Luke and Subbarao, 2006; Rudenko and Isakova-Sivak, 2015). The World Health Organization has been supporting establishment of LAIV production capacity in developing countries using the A/Leningrad/134/17/57 master donor strain as part of the pandemic preparedness plan (Friede, 2011; Rudenko et al., 2011; Surichan et al., 2011).

The production of LAIV involves growth of individual vaccine strains in embryonated eggs, partial purification of the viruses from allantoic fluid, titration of monovalent concentrated bulks and formulation into trivalent vaccine by mixing the separate strains to obtain the desired amount of infectious virus (WHO, 2013). The trivalent vaccine preparation needs to be checked for the infectivity of each strain. The titration of individual strains in the trivalent vaccine preparation is also needed for evaluating stability of the vaccine preparation after specified storage conditions.

Titration of individual strains in trivalent LAIV is usually performed by selective neutralization of two of the three strains by standard sera and end-point titration of the un-neutralized strain in embryonated eggs as EID_{50} (50% egg infectious dose) or alternatively in cell culture as $TCID_{50}$ (50% tissue culture infectious dose). This procedure requires specific sera with very high neutralizing titer against each of the vaccine strains (Yeolekar and Dhere, 2012). The standard sera are produced by immunizing specific pathogen free animals with purified hemagglutinin of the respective vaccine strains (WHO, 2013). This can be time-consuming and presents an important obstacle for vaccine production in particu-

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lar with the rapid spread of a new strain. To provide an alternative assay without the requirement of standard neutralizing sera, a new approach using subtype-specific antibodies to detect viruses in cell cultures simultaneously infected with the trivalent vaccine strains was developed.

2. Materials and methods

2.1. Monovalent and trivalent LAIV preparation

Influenza type A (H1N1 and H3N2) and type B live-attenuated reassortant vaccine strains, designated A/17/California/2009/38, A/17/Perth/09/87 and B/60/Brisbane/08/83, respectively, were obtained from the Institute of Experiment Medicine (IEM), Russian Academy of Medical Sciences, St. Petersburg, Russia. The master donor strains were used to provide six internal genes for the vaccine strains. A/Leningrad/134/17/57 (H2N2) was used for the generation of the two Influenza A H1N1 and H3N2 vaccine strains and a clone of B/USSR/60/69 for the Influenza B vaccine strain. The hemagglutinin and neuraminidase genes were from wild-type antigenic strains. Each monovalent vaccine is antigenically similar to A/California/07/09 (H1N1), A/Perth/16/08 (H3N2) and B/Brisbane/06/08. The trivalent vaccine was prepared as a mixture of the three monovalent vaccine strains.

2.2. Infectivity titration by EID_{50} assay

50% Egg Infectious Dose (EID_{50}) assay was used to determine the infectivity titers of vaccine strains in allantoic fluids of embryonated chicken eggs. Each monovalent vaccine was serially diluted ten-fold. Each dilution was inoculated into the allantoic cavity of an embryonated egg using 10 eggs per vaccine dilution. Eggs were incubated at 32° C for 72 h in egg incubator with 1 h automatic turning system and 75% humidity. After the incubation period, infected eggs were chilled at 4° C overnight before harvesting to kill the embryo and constrict the blood vessels to obtain fluids free from blood. The infectivity titers were estimated by testing the egg allantoic fluid for hemagglutination (HA) activity using the standard hemagglutination assay. Allantoic fluids were transferred into 96-well plate and then 0.5% of goose red blood cells (GRBCs) were added into each well plate. The titer was calculated using Reed and Muench method (Reed and Muench, 1938) to determine the concentrations of viral suspension that cause 50% infection of eggs. Hemagglutination assay for each test was performed in duplicate.

2.3. Subtype-specific and type-specific antibodies

The level of H1N1 infection was detected by H1-specific mouse monoclonal antibodies, ED9, while H3N2 infection was detected by H3-specific monoclonal antibodies, 12D1. The H1 and H3 specific monoclonal antibodies were obtained through the courtesy of Professor Peter Palese, Icahn School of Medicine at Mount Sinai, New York. Type specific antibody against Influenza A viral nucleoprotein, Anti-FluA-NP (#MAB8257, Millipore) and against Influenza B viral nucleoprotein, Anti-FluB-NP (#MAB8259, Millipore) were used to detect the level of influenza A infection and influenza B infection, respectively.

2.4. Infectivity titration by $TCID_{50}$ assay

The Madin-Darby Canine Kidney (MDCK) cells were used in the assay. Cells were maintained in Modified Eagle Medium (MEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 100U/ml penicillin and streptomycin (Gibco). 50% tissue culture infectious dose ($TCID_{50}$) titer was estimated for each monovalent vaccine strain in MDCK cell monolayers, with 0.25 μg/ml tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma), in 96-well plates and incubated at 33 °C, 5% CO_2 for 22–24 h. Infectivity was detected with subtype-specific anti-influenza HA antibodies, anti-influenza A or anti-influenza B nucleoprotein (NP) antibodies. The viral $TCID_{50}$ titers were calculated using Reed and Muench method.

2.5. ELISA for determining infectivity titer

Infected cells were fixed with 80% cold acetone at 4 °C for 1 h, and then blocked with 3% hydrogen peroxide, to inhibit endogenous peroxidase activity, for 30 min at room temperature. Specific anti-influenza mouse monoclonal antibody, diluted to of 1 μg/ml in blocking buffer (PBS pH 7.2 with 1% BSA and 0.1% Tween-20), was added to each well and incubated at 37 °C for 1hr, washed, followed by goat anti-mouse antibody conjugated to horseradish peroxidase (Southern Biotech Associates, USA), diluted 1:2000 in blocking buffer, and further incubated at 37 °C for 1 h, then washed. TMB peroxidase substrate system (KPL, USA) was added into each well and incubated in the dark for 10 min at room temperature. 1 M H_2SO_4 was used to stop the reaction and read at OD 450/630. Any test well with an OD > 2 times OD of cell control wells (CC) was scored positive for virus growth.

2.6. Standard sera and selective neutralization

Sheep antisera used in this study were obtained from the National Institute for Biological Standards and Control (NIBSC), UK, consisting of anti-influenza A/California/7/2009(H1N1) HA serum (NIBSC code: 11/238); anti-Influenza A/Perth/16/09 HA serum (NIBSC code: 11/206); and anti-Influenza B/Brisbane/60/08 HA serum (NIBSC code: 11/136).

The antisera were pre-treated with 3 vol of receptor destroying enzyme (RDE II) (DENKA SEIKEN,UK) at 37 °C for 18 h to remove non-specific inhibitor, followed by incubation at 56 °C for 1 h to inactivate complement. The treated antisera were then adsorbed with equal volume of 50% goose red blood cells (GRBCs) at 4 °C for 1 h to remove nonspecific agglutinins.

Effective dilutions of each antisera against each monovalent vaccine were determined. Effective dilution is the antibody titer of specific antiserum that completely neutralizes the homologous influenza virus vaccine strain by neutralization assay. The specific antiserum was 2-fold-serially diluted and mixed with fixed amount of the homologous influenza virus. The effective titer of the test antiserum is defined as the highest antiserum dilution that completely inhibits influenza hemagglutinin activity. This effective antibody titer will be further used in the experiment on determining the potency of individual vaccine component present in a dose of trivalent live attenuated influenza vaccine based on blocking the infectivity of the other two influenza vaccine strains with specific antisera, and followed by determining for the infective titers of the remaining non-neutralized strain.

Three mixtures of Anti (H1 + H3), Anti (H1 + B) and Anti (H3 + B) were prepared at a concentration of five times the effective dilution. Selective neutralization was carried out by mixing trivalent vaccine with each mixture of antisera. The titer of the remaining strain that has not been neutralized was measured using the previously described ELISA and estimated using Reed and Muench method.

2.7. Titration of vaccine strains in trivalent LAIV preparation

$TCID_{50}$ titers of individual vaccine strain in trivalent vaccine were determined by ELISA using specific antibody without prior neutralization by antisera mixtures. The trivalent vaccine preparation was serially diluted and inoculated on to MDCK cell monolayer in 96-well micro culture plate, then incubated at 33 °C, 5% CO_2 for