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Implementation of new approaches for generating conventional reassortants for live attenuated influenza vaccine based on Russian master donor viruses

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Abstract

Cold-adapted influenza strains A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69, originally developed in Russia, have been reliable master donors of attenuation for preparing live attenuated influenza vaccines (LAIV). The classical strategy for generating LAIV reassortants is robust, but has some disadvantages. The generation of reassortants requires at least 3 passages under selective conditions after co-infection; each of these selective passages takes six days. Screening the reassortants for a genomic composition traditionally starts after a second limiting dilution cloning procedure, and the number of suitable reassortants is limited. We developed a new approach to shorten process of preparing LAIV seed viruses. Introducing the genotyping of reassortants by pyrosequencing and monitoring sequence integrity of surface antigens starting at the first selective passage allowed specific selection of suitable reassortants for the next cloning procedure and also eliminate one of the group selective passage in vaccine candidate generation. Homogeneity analysis confirmed that reducing the number of selective passages didn't affect the quality of LAIV seed viruses. Finally, the two-way hemagglutination inhibition test, implemented for all the final seed viruses, confirmed that any amino acid substitutions acquired by reassortants during egg propagation didn't affect antigenicity of the vaccine. Our new strategy reduces the time required to generate a vaccine and was used to generate seasonal LAIVs candidates for the 2012/2013, 2014/2015, and 2015/2016 seasons more rapidly.

Keywords

Influenza; Live vaccine; Reassortants generation

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Conflict of interest

None.

1. Introduction

Vaccination is the most effective method for preventing influenza virus infection (Fiore et al., 2009). Influenza vaccines are most protective when the component strains antigenically match viruses circulating in the population. Seasonal influenza vaccines contain influenza A/H1N1, A/H3N2, and 2 types of influenza B virus, Victoria and Yamagata lineage. In addition, vaccine seed strains against avian H5, H7, and H9 viruses are produced for pandemic preparedness purposes.

The two major types of influenza vaccines licensed for human use are: inactivated influenza vaccine, which is injected, and live attenuated influenza vaccine (LAIV), which is administered intranasally. The LAIV seed viruses are reassortants, which supply the appropriate surface protein hemagglutinin (HA) and neuraminidase (NA) genes from seasonal isolates, and internal genes from cold adapted type A or type B master donor viruses (MDV), which provide cold adaptation, temperature sensitivity, and attenuation phenotypes. These phenotypic characteristics enable the LAIV reassortants to replicate efficiently in cooler temperatures, restrict replication to the upper respiratory tract and attenuate the virus (Maassab and Bryant, 1999; Murphy and Coelingh, 2002). LAIVs confer protection by inducing the development of neutralizing anti-HA antibodies, (Belshe et al., 2000; Cox et al., 2004; Gerhard, 2001), mucosal cellular responses and can provide heterosubtypic protection (Epstein and Price, 2010; Haaheim and Katz, 2011; He et al., 2006; Hoft et al., 2011).

LAIVs have been an effective public health tool for years in the Russian Federation (Aleksandrova, 1977; Rudenko et al., 1996; Smorodintsen et al., 1965) and the USA (Ambrose et al., 2008; Bandell et al., 2011; Maassab and Bryant, 1999; Murphy and Coelingh, 2002). Two types of LAIVs are available commercially. The first, licensed as FluMist (MedImmune, Inc.), is based on A/Ann Arbor/6/60 influenza A and B/Ann Arbor/1/66 influenza B; it is currently produced using seed viruses made by reverse genetics (Ambrose et al., 2008; Jin and Chen, 2014). The LAIVs based on Russian MDV strains are made using seed viruses produced by conventional reassortment in eggs (Aleksandrova, 1977; Kiseleva et al., 2007, 2014). Russian LAIVs were developed and used safely for more than 50 years in Russia, and recently, through cooperation with WHO, their production and use has been expanded internationally (Neuzil et al., 2012; Rudenko et al., 2011). The preferable method approved by WHO for seasonal LAIV seed viruses candidates preparation is reassortment in eggs, due to intellectual property issues currently present for reverse genetics generated LAIV vaccines, the production of such vaccines could be costly, which is a concern for developing countries manufacturers (http://www.who.int/phi/Day1_Session3_PATH_Marks.pdf).

The protocol for generating LAIVs based on Russian MDVs by conventional reassortment was originally developed at the Institute of Experimental Medicine (IEM), St. Petersburg, Russia. The protocol includes 2 selective passages and 1 cloning step performed at selective conditions, in the presence of anti-serum against MDV and at low temperature (25 °C) to allow the correct 6:2 reassortants to dominate the pool. However, even under such robust selective pressure, variability of the gene segments donated by the cold-adapted donor in the

vaccine reassortants was often observed (Ghendon et al., 1981; Kiseleva et al., 2007, 2014; Medvedeva et al., 1983). It was also noted that most viruses circulating prior to the year 2000 readily reassorted with MDVs; in contrast, vaccine seed strains based on circulating human viruses after the year 2000 were much more difficult to obtain. Only 11–14% of reassortants derived from post-2000 viruses using standard techniques possessed the desired NA, and the percentage of M and NS gene segments derived from MDVs was also relatively low (Kiseleva et al., 2014). The obstacles in developing current LAIV candidate vaccine seeds prompted us to optimize the current protocol to increase the yield of the desired 6:2 reassortants and expedite the LAIV seed virus production without compromising the quality of the product.

2. Materials and methods

2.1. Viruses

A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69 MDV were provided by BioDiem (Australia). Seasonal influenza isolates propagated in 9- to 11-day-old embryonated eggs A/Texas/50/2012 (H3N2), B/Massachusetts/02/2012 (Yamagata), A/Anhui/01/2013 (H7N9), B/Texas/02/2013 (Victoria), A/Palau/6759/2014 (H3N2), A/Switzerland/9715293/2013 (H3N2), B/Phuket/3073/2013 (Yamagata), and A/North Carolina/13/2014 (H3N2) were obtained from CDC, Atlanta, USA. For vaccine preparation specific pathogen free (SPF) eggs (Charles River Laboratories Inc., USA) were used.

2.2. The conventional IEM method of reassortment

The eggs were co-inoculated with equal doses (6 $\log_{10}$ of EID_{50}) of MDV and seasonal isolate. Co-inoculated viruses allowed to propagate and reassort at 32 °C for 48 or 72 h for type A and type B influenza, respectively. The progeny of the co-inoculation subjected further to the two group selective passages and one cloning by limiting dilution procedure under selective condition—temperature of 25 °C and the presence of serum against MDV. Usually, five chicken eggs are inoculated with pooled allantoic fluids from the co-inoculation step, which was incubated overnight with antisera against MDV. A serum dilution with a final HI titer of 1:60 (experimentally determined to efficiently neutralize MDV but not viruses containing the desired parental HA) was used. The presence of virus in the progeny of infected eggs can be detected by the HA assay after each step of vaccine generation. If HA titer could not be detected after selective passaging at 25 °C, then a “blind” passage (32 °C without serum, 2 days for influenza type A, or 3 days for type B) was performed. After the blind passage the progenies from all the positive eggs pooled again and used to infect eggs at the same conditions (second selective passage). The progeny from the second selective is pooled and then serially diluted 10-fold (from 10^{-1} to 10^{-7}) using serum against the MDV as a diluent (five eggs were inoculated with each virus dilution) and incubated at 25 °C for 6 days. From the eggs infected with the last positive limiting dilution with detectable HA titers 2–3 eggs are selected. The second cloning takes place at 32 °C in the presence of anti-MDV serum. The cloned progeny is then genotyped to find the reassortants containing the 6:2 genome composition (Desheva Iu et al., 2007; Kiseleva et al., 2011a,b). After a 6:2 reassortant is found, additional rounds of cloning are performed in order to enhance the purity of the reassortant virus progeny. Usually, the number of 6:2

reassortants is very limited. If no 6:2 reassortants are detected after the second cloning, reassortment must be started anew.

2.3. Hemagglutination (HA) and hemagglutination inhibition (HI)

HA and HI assays were performed using 0.5% turkey or 0.75% guinea pig red blood cells (Klimov et al., 2012; WHO, 2011). Ferret serum against MDV and parental influenza isolates was raised and used in HI to determine the origin of the HA protein in the reassortant clones.

2.4. Two-way HI test

To determine the antigenic relationship between the parental reference virus and the LAIV reassortants generated, ferret antisera were raised against both parental contemporary human isolates and candidate LAIV reassortants for use in the HI analysis (WHO, 2011). Reassortant viruses were considered consistent with parental isolate if the heterologous titer was within a 2-fold of the homologous HI titer of the parental reference isolate.

2.5. Genotyping of reassortants

Genomic composition of the reassortant influenza viruses was assessed by pyrosequencing as described previously (Shcherbik et al., 2014b). Briefly, cDNA was produced from viral RNA with gene specific sets of primers in which either the forward or reverse primer was biotinylated. The biotinylated amplicons were bound to streptavidin-coated beads and subjected to denaturation and washing to generate single stranded DNAs, which were then annealed with pyrosequencing primer, designed to detect the strain-specific signature nucleotides. Pyrosequencing reactions were performed with the PyroMark Gold enzyme, substrate and nucleotides using the PyroMark ID instrument (Qiagen). Sequence results were obtained in the form of pyrograms and analyzed using visual interpretation and the PyroMark Q96 software (Qiagen).

2.6. Genetic homogeneity analysis

Genetic homogeneity analysis of the LAIV seed viruses was performed using real time RT-PCR as described previously (Shcherbik et al., 2014a).

2.7. Genomic analysis

Complete cDNAs for each segment (PB2, PB1, PA, HA, NP, NA, M, and NS) of the LAIV candidates were reverse transcribed using gene segment-specific primers and sequenced by standard Sanger technique. The sequences of HA and NA genes were deposited in the influenza sequence EpiFlu Database of GISAID (http://platform.gisaid.org). Accession numbers are: for A/Anhui/1/2013-CDC-LV7A (H7N9), EPI516488 and EPI516487; for B/Texas/02/2013-CDC-LV8B (Victoria lineage), EPI516490 and EPI516489; for A/Switzerland/9715293/2013-CDC-LV10A (H3N2), EPI543739 and EPI543740; for B/Phuket/3073/2013-CDC-LV11B (Yamagata), EPI573303 and EPI573304; for A/North Carolina/13/2014-CDC-LV12A (H3N2), EPI573305 and EPI573306.

3. Results

3.1. Screening LAIV reassortants at each step of vaccine generation

The generation of 6:2 LAIV reassortants by traditional IEM protocol (described in Materials and Methods) from co-infection to genotyping after second cloning procedure takes usually 32 days for influenza A and 39 days for influenza B (Table 1). In order to improve and accelerate the process of generation of reassortants with the suitable genomic composition, several changes to the IEM protocol were introduced. The screening analysis was performed at each step of vaccine generation starting from the first selective passage in order to choose the most suitable candidates for the next limiting dilution cloning procedure. The sequencing of HA gene of LAIV reassortants at each cloning step of vaccine generation was also introduced to monitor undesired changes.

The reassortment between MDV-A and A/North Car-olina/13/2014 (H3N2) (NC/13) an A/Switzerland/9715293/2013-like virus, the vaccine candidate recommended by WHO for the 2015–2016 season in the Northern hemisphere, is presented as an example. The eggs were co-infected with equal doses of each virus. The progeny of the co-infection was subjected to the first selective passage in the presence of anti-MDV-A sera at 25 °C for 6 days. Since no HA activity was detected after the first selective passage, a blind passage was done at 32 °C for 2 days. The progeny of the blind passage were then analyzed using the HI assay with sera raised against parental NC/13 virus or the MDV. Progeny from 5/5 of the inoculated eggs showed inhibition by sera against parental but not by sera against the MDV, indicating that the HA gene segments of the progeny virus were predominantly of the desired parental NC/13 origin.

Following the traditional IEM protocol, we would combine all the progeny from the first selective passage to infect five new eggs for a second selective passage. Using our modified protocol, however, we screened the progeny harvested from individual eggs after the first passage using the pyrosequencing assay. Pyrosequencing is sequencing by synthesis method, where the peak heights in the pyrograms reflects the number of each nucleotide incorporated and “signature peaks” represent the percentage of each cDNA species present in the mixture. Partial sequences of RT-PCR amplicon’s of RNA isolated from allantoic fluid after the first selective passage were obtained for all genes and showed that NC/13 NA was prevalent in all eggs (Fig. 1A), while PB2, PB1, PA, NP, and M were present in a mixed population (PB2 gene is shown in Fig. 1B). The NS gene segment was predominantly of undesired NC/13 origin in the reassortants from all eggs (Fig. 1C). However, the progeny of egg 21 had the highest enrichment of the desired origins of all internal virus genes segments (e.g., PB2 and NS pyrograms Fig. 1).

The progeny of egg 21 was subjected to the first cloning step under selective conditions (serum to MDV, 25 °C), omitting the second selective passage. Screening revealed that all 15 clones from the first cloning of egg 21 had the correct NC/13 origin for NA gene segment. One biological clone was the correct 6:2 reassortant, and another had a 5:3 genome composition (with HA, NA, and M originating from the NC/13 virus). All other clones had a mixed populations for internal protein coding gene segments, with MDV gene segments (including NS) prevailing over NC/13 gene segments in 7 clones. If we had not detected a

6:2 reassortant at this step, one of the clones with a higher proportion of MDV gene segments would have been selected for further cloning by limiting dilution until a 6:2 reassortant could be identified. The selected 6:2 reassortant was subjected to two additional rounds of cloning procedure followed by the amplification step. We successfully used this strategy to generate eight LAIV seed viruses listed in Table 3.

In parallel, a second selective passage (a step required by the traditional IEM protocol) was also performed on the progeny from the first selective passage of the above example. Analysis of the progeny from the second selective passage revealed that most of the internal gene segments (PA, PB1, PB2, M, and NP) were of MDV origin (PB2 gene is shown in Fig. 2A; compare peaks marked by green arrows in 2A and 1B). However, the amount of the desired MDV NS gene was lower than in reassortants from the first selective passage (Fig. 2B; compare peaks marked by green arrows in Figs. 1C and 2B), illustrating that biological cloning from selected eggs after the first selective passage not only saves time but increases one's ability to select for the desired reassortant. As recommended by the current IEM protocol, the progeny from the second selective passage were combined, and cloning by endpoint dilution under selective conditions (25 °C, serum to MDV) was performed. Genotyping the clones obtained showed that the internal gene segments were still a mixed population, with NC/13 NS gene segment prevalent in all clones. These demonstrated that the second selective passage does not ensure higher yields of the desired internal gene segments. Similar results were observed when we generated the LAIV candidate for A/Victoria/361/2011-CDC-LV1 (H3N2) where after the second selective passage none of the clones from the second cloning step had the desired NS gene segment of MDV origin. Likewise, during generation of B/Texas/6/2011-CDC-LV2B (Yamagata lineage), none of the clones analyzed had NA of the desired parental contemporary virus origin.

Our results showed that genotyping at each step of vaccine generation, starting from the first selective passage to select the best candidate for the subsequent cloning procedure, allowed us to omit the second selective passage from the protocol and to accelerate the generation of the desired 6:2 LAIV seed virus (Table 1).

3.2. Sequencing the HA gene of LAIV reassortants at each cloning step of vaccine generation

The first virus cloning step in our modified LAIV vaccine candidates generation strategy enables selection of several virus progenies suitable for the subsequent cloning procedure, therefore, sequencing the HA and NA gene segments of selected clones should be considered at this step to determine the most appropriate candidates for further cloning. As an example from our studies, during generation of LAIV seed virus for A/Texas/50/2012 (H3N2), we identified 4 clones possessing desired HA and NA gene segments and high proportions of internal MDV gene segments. Progeny of egg 4171 was the best candidate for further cloning as one with the highest proportion of MDV internal gene segments. However, sequencing revealed that candidate 4171 had a H156Q substitution in HA sequence (Table 2); this substitution known to affect antigenicity of other H3N2 influenza viruses and vaccines (Barr et al., 2014; Kodihalli et al., 1995; Skowronski et al., 2014). Instead, candidate 4162, which had the egg-adapted mutation I226N (not known to affect

antigenicity) was selected for further cloning to obtain genetically homogeneous 6:2 reassortants. The HA and NA gene segments were sequenced at each limiting dilution step to verify the lack of amino acid substitutions in these gene segments.

The amino acid changes in HA and NA proteins of the LAIV seed viruses generated in the present study are summarized in Table 2. None of the reassortants had amino acid changes in the internal segments originating from MDV. Additionally, all LAIV reassortants showed high virus titer at the optimal temperature of 32 °C, very low titer at 38–39 °C, and less than 3 $\log_{10}$-fold decrease in replication at 25 °C compared to 32 °C, confirming their temperature-sensitive and cold-adapted phenotypes (Table 3).

3.3. Quality control of the LAIV seed viruses

The reassortant virus has to be fully characterized in order to become a master seed virus for vaccine production, WHO outlines the requirements for complete characterization of live attenuated vaccine master seed virus (WHO, 2013). To ensure that the quality of the LAIV seed viruses obtained using our alternative reassortment strategy was not compromised, we used real-time RT-PCR to test homogeneity of each reassortant (Shcherbik et al., 2014a). Genetic homogeneity of reassortant viruses listed in Table 3 were analyzed using RNA isolated from progeny of infected eggs corresponding to 10^5 EID_{50}/per reaction. For all reassortants, the positive signals were detected only with primers/probe specific for internal gene segments of MDV and HA and NA gene segments of desired parental viruses (data not shown). During vaccine manufacturing, the number of passages the LAIV candidate virus undergoes should not exceed 4–5 (Buonagurio et al., 2006). The desired 6:2 genome composition of the LAIV seed viruses was confirmed after 5 more passages in eggs using real time RT-PCR and complete genome sequence analysis. Sequencing revealed no nucleotide substitutions in all gene segments during these additional passages in all tested seed viruses. Thus, the genomic composition of all the LAIV seed viruses generated (Table 3) remained stable even after additional passaging in hen's eggs.

To ensure that these mutations acquired during LAIV generation do not alter antigenicity, two-way HI assays have been implemented as a mandatory requirement for characterization of vaccine seed viruses. That is, the reassortant should be strongly inhibited by serum raised against the contemporary parental virus, and the same parental virus should be strongly inhibited by ferret serum raised against the LAIV virus. Reassortant viruses are considered consistent with the parental reference virus antigenically if the heterologous HI titer is within a 2-fold difference of the homologous HI titer (Barr et al., 2010; Katz et al., 2011). All of the LAIV seed viruses listed in Table 3 were antigenically consistent with their parental reference viruses, and therefore successfully passed the two-way HI tests.

4. Discussion

The conventional reassortment procedure was developed and successfully used for years at the IEM, Russia, to make LAIV seed reassortants (Aleksandrova, 1977; Medvedeva et al., 1983; Polezhaev et al., 1978). The traditional procedure for LAIV generation is robust but has some disadvantages. It is time-consuming, as the selection steps are lengthy. The genome composition of the reassortants is only screened after the second cloning procedure,

Genome combinations other than the 6:2 dominate in the reassortant populations of LAIV seed viruses prepared using the traditional scheme (Kiseleva et al., 2007), especially for viruses that emerged after the year 2000 (Kiseleva et al., 2014). Another drawback of the protocol is the lack of HA sequence monitoring during LAIV generation, since additional egg-adapted mutations can accumulate in the HA gene which may affect antigenicity of the final reassortant virus.

To reduce the duration of LAIV seed virus generation and improve the yield of 6:2 reassortants without affecting antigenicity, we considered a few steps in the protocol for possible alteration. Here, we demonstrate that genotyping the reassortants at earlier stages of LAIV preparation is an important advantage over the current procedure. Genotyping by pyrosequencing is semi-quantitative and allows the assessment of the relative amounts of gene segments of the desired origin in allantoic fluid (Shcherbik et al., 2014b). This enables rapid selection of desired reassortants at earlier stages of the process. Our results demonstrate that the second selective step in the procedure could be eliminated since it does not ensure higher yields of desired gene segments (Fig. 2), and this could saves up to two weeks in a demanding vaccine preparation process (Table 1).

Monitoring the virus sequences for changes which could be introduced during egg-based vaccine production is also critical for vaccine effectiveness. Often egg adaptation of human influenza viruses results in amino acid changes in or near receptor-binding site, which is located on the head region of the HA glycoprotein. Some egg adaptations dramatically alter virus antigenicity, immunogenicity, and vaccine efficacy (Chen et al., 2010; Katz and Webster, 1989; Kodihalli et al., 1995; Skowronski et al., 2014). Low effectiveness of seasonal influenza vaccines has been attributable to mutations associated with egg adaptation (Kishida et al., 2012; Skowronski et al., 2014). The mutations can appear at the initial stages of candidate vaccine seed virus generation, or even before, since egg-propagated isolates are used for seed virus generation. Monitoring the sequences of the surface antigen gene segments at every step of the reassortment process, starting at the first selective passage, allows for rapid selection of clones with minimal number of mutations, while discarding clones which have mutations known to affect antigenicity. Antigenic identity determined by two-way HI assay is currently widely accepted for vaccine candidate characterization (Barr et al., 2010, 2014; Katz et al., 2011). All candidate vaccine reassortants listed on WHO web site as recommended for use in the current influenza season (http://www.who.int/influenza/vaccines/virus/en/) passed the two-way HI test. Successful results of the two-way HI assay for the final seed virus confirm that any mutations acquired by the reassortant during egg propagation do not affect antigenicity, and that a particular candidate vaccine seed can be recommended for vaccine production.

One of the major concerns in LAIV production by conventional reassortment is homogeneity. Since LAIV is produced by biological cloning using limiting dilution in eggs, there is a possibility of obtaining an impure vaccine candidate. To ensure that the final candidate vaccine seed virus contains a pure genomic constellation, we developed a real-time RT-PCR assay (Shcherbik et al., 2014a) and used it to examine all LAIV candidate reassortants. In addition, we subjected each seed virus to 5 additional egg passages followed by sequence analysis and real-time RT-PCR to demonstrate the stability of the vaccine seeds

genome composition and sequence integrity. Sequencing the reassortants after these passages did not detect any changes in the genomes of any of the reassortants tested, confirming their genetic stability and suitability as vaccine seed viruses for manufacturing.

In conclusion, our study demonstrates that the improved protocol has certain advantages over the conventional IEM protocol—we were able to shorten the duration of LAIV generation, precisely select reassortants with the desired 6:2 genome composition and without egg-adaptive mutations that often negatively impact antigenicity. Our new strategy was successfully used to develop LAIV seed viruses for the 2013/2014, 2014/2015, and 2015/2016 influenza seasons.

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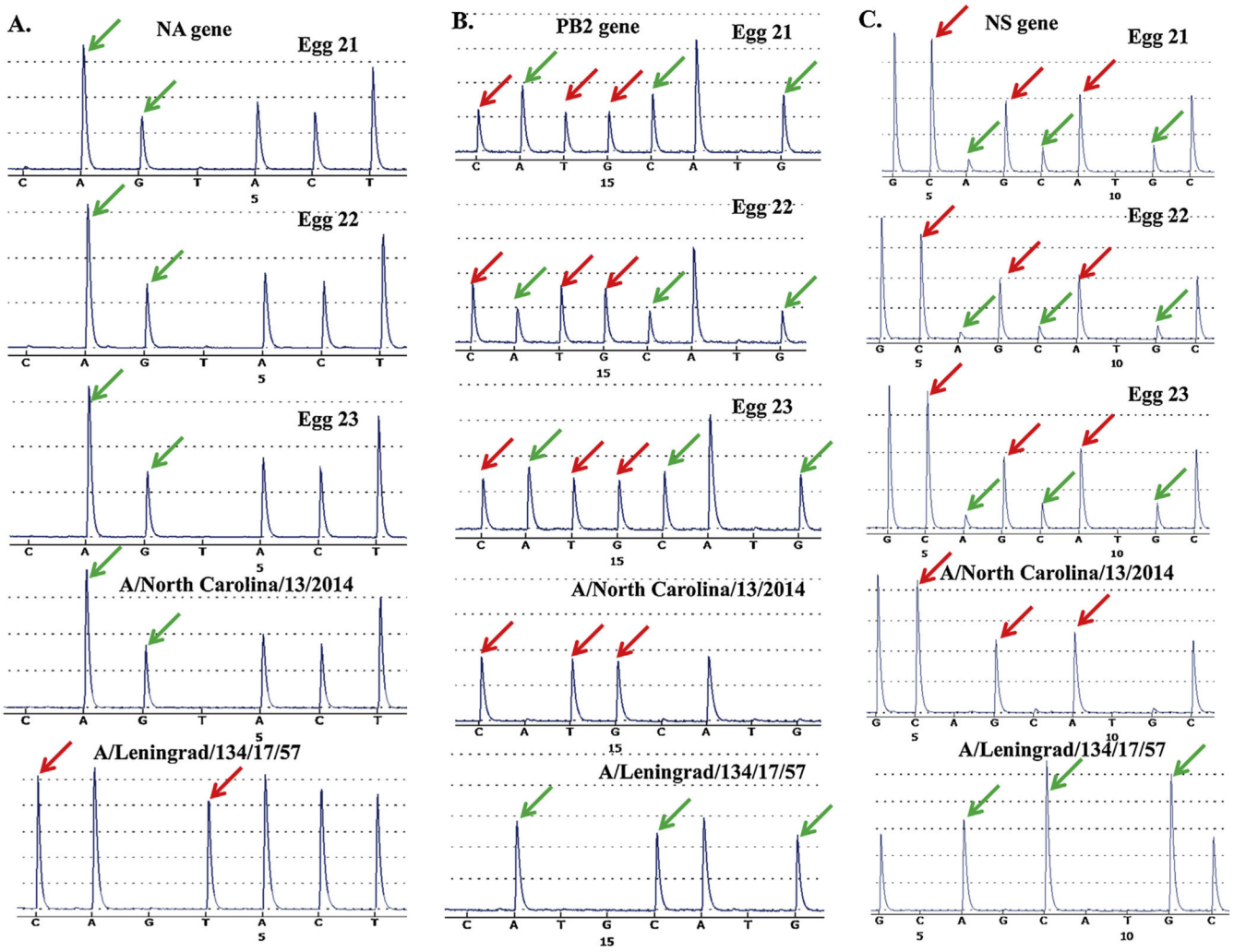

Fig. 1.

Partial pyrograms of neuraminidase (NA) (A), PB2 (B), and NS (C) RNA from eggs after the first selective passage (eggs 21, 22, and 23 are shown) after co-infection of A/North Carolina/13/2014 and MDV. Green arrows indicate signature peaks of desired origin (NA—A/North Carolina/13/2014, internal genes—MDV), red arrows indicate signature peaks of genes on undesired origin in a mixed reassortants pool.

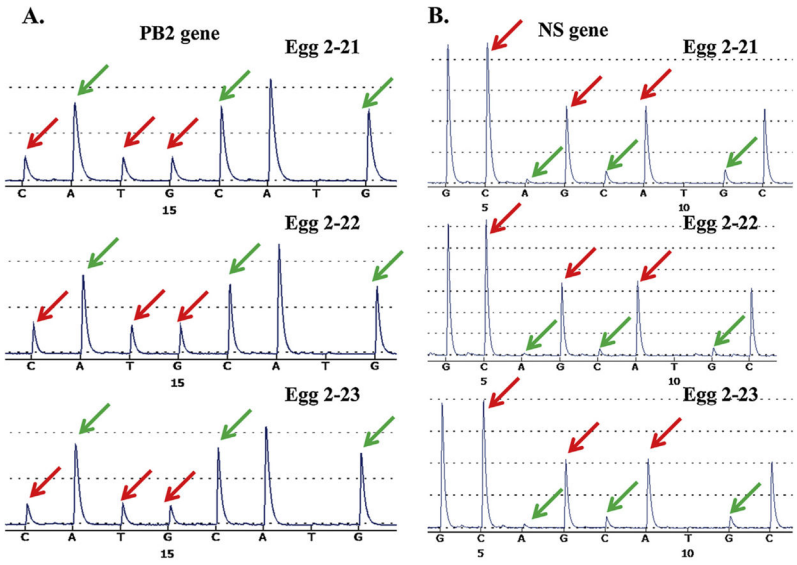

Fig. 2.

Partial pyrograms of PB2 (A) and NS (B) gene RNA from eggs after the second selective passage (eggs 2–21, 2–22, and 2–23) after co-infection of A/North Carolina/13/2014 and MDV. Green arrows indicate signature peaks of desired origin (MDV), red arrows indicate signature peaks of A/North Carolina/13/2014 in a mixed reassortants pool.

Table 1

Time flow of LAIV seed virus generation and techniques used for selection of reassortants.

Steps in IEM protocol	Techniques used in IEM protocol	Steps in modified protocol	Techniques used in modified protocol
1. Co-infection of WT and MDV, 32 °C (2 or 3 days)		1. Co-infection of WT and MDV, 32 °C (2 or 3 days)	
2. Selective passage 1, 25 °C (6 days)		2. Selective passage 1, 25 °C (6 days)	
3. Blind passage, 32 °C (2 or 3 days)		3. Blind passage, 32 °C (2 or 3 days)	3a. HI assay for detection of HA origin, pyrosequencing of other genes, HA, NA sequencing; selection of the egg(s) with the highest enrichment of the desired genes and suitable HA, NA sequences
4. Selective passage 2, 25 °C (6 days)			
5. Blind passage, 32 °C (2 or 3 days)			
6. First cloning 25 °C, (6 days)		4. First cloning, 25 °C (6 days)	
7. Blind passage, 32 °C (2 or 3 days)	7a. High growth reassortant selection by HA assay, HI assay for detection of HA origin, selection of egg(s) for further cloning	5. Blind passage, 32 °C (2 or 3 days)	5a. Pyrosequencing, HA, NA sequencing, selection of the egg(s) with the highest enrichment of the desired genes
8. Second cloning, 32 °C (2 or 3 days)	8a. HA origin by HI assay, genotyping other genes by RT-PCR RFLP, selection of 6:2 reassortant	6. Second cloning, 32 °C (2 or 3 days)	6a. Pyrosequencing, HA, NA sequencing, selection of 6:2 reassortant
9. Third and fourth cloning 32 °C, (4 or 6 days total)		7. Third and fourth cloning, 32 °C (4 or 6 days total)	7a. HA, NA sequencing at each cloning step
10. Amplification Influenza A 32 days Influenza B 39 days	10a. Full genome sequencing	8. Amplification Influenza A 24 days Influenza B 30 days	8a. Full genome sequencing

Table 2

Amino acid changes in HA and NA of intermediate clones during the generation of A/Texas/50/2012-CDC-LV6A.

Clone no.	**4162**	**4163**	**4171**	**4186**
HA changes	I226N	I226(I/N)	H156Q	I226N
NA changes	S334T	None	None	None

Table 3

Genetic and phenotypic characteristics of LAIV seed viruses.

Vaccine seed virus	HA changes	NA changes	log_{10} EID_{50}/mL 32 °C	log_{10} EID_{50}/mL 25 °C	log_{10} EID_{50}/mL 38 °C
B/Massachusetts/02/2012-CDC-LV5B (Yamagata lineage)	None	None	9.5	6.2	0
A/Texas/50/2012-CDC-LV6A (H3N2)	I226N	S334T	9.2	6.2	0
A/Anhui/01/2013-CDC-LV7A (H7N9)	N123D, N149D*	T10I	9.9	6.4	2.7
B/Texas/02/2013-CDC-LV8B (Victoria lineage)	I197N	None	9.5	6.3	0
A/Palau/6759/2014-CDC-LV9A (H3N2)	S219Y	None	9.3	6.2	2.0
A/Switzerland/9715293/2013-CDC-LV10A (H3N2)	S219Y	None	8.7	5.7	1.7
B/Phuket/3073/2013-CDC-LV11B (Yamagata lineage)	N197D	None	8.5	5.9	0
A/North Carolina/13/2014-CDC-LV12A (H3N2)	S219F	None	9.5	6.4	0

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