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EXPERIMENTAL WORKS

Genome Composition Analysis of Reassortant Influenza Viruses Used in Seasonal and Pandemic Live Attenuated Influenza Vaccine[1]

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Abstract—The cold-adapted, temperature sensitive and attenuated influenza master donor viruses A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69 were used to generate vaccine viruses to be included in live attenuated influenza vaccine. These vaccine viruses typically are 6 : 2 reassortant viruses containing the gene segments of the surface antigens haemagglutinin and neuraminidase of current wild type influenza A and influenza B viruses with the gene segments encoding the internal viral proteins, conferring the cold-adapted, temperature sensitive and attenuated phenotype, being inherited from the master donor viruses. The 6 : 2 reassortant viruses are selected from co-infections between master donor virus and wild type viruses that theoretically may yield as many as 256 combinations of gene segments and thus 256 genetically different viruses. As the time to generate and isolate vaccine viruses is limited and because only 6 : 2 reassortant viruses are allowed as vaccine viruses, sboth selection and creening needs to be both rapid and unambiguous. The screening of reassortant viruses by RT-PCRs using master donor virus and wild type virus specific primer sets is described to select both influenza A and influenza B 6 : 2 reassortant viruses to be used in seasonal and pandemic live attenuated vaccine, unambigously.

Keywords: influenza virus, genome composition analysis, reassortants, live influenza vaccine.
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Live attenuated influenza vaccine (LAIV) is in use in Russia for decades and since 2003 in the United States of America. The Russian LAIV is based on the master donor viruses (MDVs) A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69 which are cold-adapted, temperature sensitive and attenuated [1, 6, 8]. LAIV has a long record of being safe and efficacious. The safety of LAIV is guaranteed by its cold-adapted, temperature sensitive and attenuated phenotype which limits viral replication to the upper respiratory tract where the vaccine is administered. Efficacy is achieved by induction of local and systemic humoral and cellular immune responses accomplished by mimicking natural infection.

The segmented nature of the influenza virus genome enables exchange of each of eight gene segments between two viruses following dual infection. This process is referred to as reassortment and the resulting viruses are called reassortant viruses. Reassortment is exploited in generating attenuated vaccine viruses to be used in LAIV. Circulating wild type influenza A or B virus recommended by WHO for use in current epidemic season as vaccine candidates are co-infected with MDVs. The sole one of interest is a so called 6 : 2 reassortant virus which contains the gene segments encoding the surface glycoproteins—haemagglutinin (HA) and neuraminidase (NA)—of the wild type virus to be represented in the vaccine while the remaining gene segments, which encode the internal viral proteins, including those conferring attenuation, must originate from the MDV.

Two cold-adapted viruses, A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69, were generated in 1960s–1970s in the Department of Virology named by Acad. A. A. Smorodintsev (Institute of Experimental Medicine, North-West Division of the Russian Academy of Medical Sciences) and now they are the only MDVs approved in Russia for development of Russian live attenuated influenza vaccine strains.

Co-infections typically yield numerous genetically different viruses. Indeed, theoretically a total of 256 (2^8) different combinations of gene segments and, consequently, an equal number of different viruses may arise following co-infection. To diminish the number of undesired reassortant viruses arising during

[1] The article was translated by the authors.

co–infection, antiserum raised against the MDV and low temperature of incubation are being used in subsequent selective passages. Since time to generate vaccine viruses is limited and only 6 : 2 reassortant viruses are acceptable for inclusion in LAIV, a rapid, unambiguous and reliable method to screen reassortant viruses is of utmost importance.

Here, we describe RT-PCR methods using gene segment and virus (sub)type specific primers allowing for rapid and unequivocal screening of reassortant influenza A and influenza B viruses to be used in seasonal and pandemic LAIV.

MATERIALS AND METHODS

Viruses. Influenza wild type viruses were obtained from CDC (Atlanta, GA) and from the National Institute for Biological Standards and Control (NIBSC, Potters Bar, UK). The MDVs A/Leningrad/134/17/57 (H2N2) and B/USSR/60/69 were obtained from the Institute of Experimental Medicine (Saint Petersburg, Russia). Viruses were propagated in 10 to 11 day old embryonated hen's eggs. Reassortant viruses were generated by routine technique in embryonated chicken eggs or Madin–Darby Canine Kidney (MDCK) cells (ATCC, Cat. No. CCL-34) as described in [18]. Briefly, equal doses of MDV and wild type virus were inoculated into eggs or cells and incubated at optimum temperature of 32°C (co-infection). The development of vaccine reassortants involved sequential passages of reassortant progeny at 25°C in the presence of antiserum to MDV and by limit dilution cloning in the presence of antiserum to MDV.

RNA isolation. RNA was isolated from influenza virus infected allantoic fluid or cell culture supernatant using the QIAamp Viral RNA Minikit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany).

cDNA synthesis. cDNAs were synthesized using commercially available Quantitect Reverse Transcription Kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). For cDNA synthesis universal primers were used so that in a single reaction cDNAs were generated for all eight gene segments. These cDNAs were used as templates in the PCRs. The universal forward primer (Invitrogen) used for influenza A viruses was AUNIVERSALF (5'TCGACGTCTCAGGGAGCAAAAGCAGG). The universal forward primer (Invitrogen) used for influenza B viruses was BUNIVERSALF (5'ACGTTGCAAGCAGAAGC). The thermal cycler program for the reverse transcription (RT) reaction was as follows: 42°C for 2 min: (put on ice while adding next step): 42°C for 15 min: 95°C for 3 min.

Design of PCR programs and selection of primers for screening of reassortant influenza A and B viruses. PCR programs and primers were designed such that clear PCR products were obtained for the gene segments of one of the parental viruses used in co-infection (MDV or wild type virus) while under the same PCR conditions and using the same primers no PCR products were obtained for the other parental virus. Gene segment and virus (sub)type specific primers were selected in such a way that PCR products of different sizes are obtained for wild type virus and MDV specific primers and that wild type virus specific primers do not amplify MDV gene segments and vice versa. Twelve PCR programs that were used for genotyping of reassortant viruses in order to select 6 : 2 vaccine viruses are presented in Table 1. Sequences of each of the gene segments of wild type influenza A viruses as well as wild type influenza B viruses were aligned with those of the MDVs A/Leningrad/134/ 17/57 (H2N2) and B/USSR/60/69, respectively.

Sequences of wild type viruses were obtained from sequence databases (http://www.flu.lanl.gov and http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/select.cgi). Multiple sequence alignment analysis was performed using Clone Manager 9.

PCR and gel electrophoresis. PCRs were carried out in Eppendorf Master Cycler® gradient. Amplified products were detected by 0.5 μg/mL ethidium bromide staining of amplified products after 1.7% agarose gel electrophoresis.

All reagents were obtained from SibEnzime (Novosibirsk, Russia).

RESULTS AND DISCUSSION

Because of high antigenic variability of influenza viruses, the composition of influenza vaccines for use in each influenza season is changed annually. This means that for LAIV, vaccine strains have to be made annually as well by classical re-assortant techniques. Selection and screening of reassortant viruses for their genetic make-up preferably has to be convenient and rapid. Therefore, time for screening and characterizatioin of reassortant vaccine strains for any new epidemic season is limited; the efficacy of development of vaccine depends on the method of identification and analyses of its genome composition. The final step of screening modern live influenza vaccine candidates is full genetic sequencing, but at the intermediate steps when a large number of reassortant viruses have to be urgently analyzed there is need for a rapid and reliable method of primary screening.

Determination of the origin of gene segments in reassortant influenza viruses can be done using several techniques. Polyacrylamide gel electrophoresis of viral

Table 1. Programs for comparative PCR analysis of genome composition of reassortants of master donor viruses with wild type viruses

Program code	Preheating		PCR main stages							Post-PCR	
			Melting		Annealing		Polymerization		No. of cycles		
	time, min	temp, °C	time, s	temp, °C	time	temp, °C	time, min	temp, °C		time, min	temp, °C
1	4	94	30	94	30 s	55	1	68	30	7	68
2	4	94	30	94	30 s	60	1	72	30	7	72
3	4	94	30	94	30 s	63	1	72	30	7	72
4	4	94	30	94	30 s	55	1	72	30	7	72
5	4	94	30	94	1 min	55	1	68	45	7	68
6	4	94	30	94	1 min	55	1	68	30	7	68
7	4	94	30	94	1 min	63	1	68	30	7	68
8	4	94	30	94	1 min	61	1	68	45	7	68
9	4	94	30	94	1 min	50	1	68	45	7	68
10	4	94	30	94	1 min	61	1	68	30	7	68
11	4	94	30	94	1 min	50	1	68	30	7	68
12*	4	94	30	94	30 sec	48	1	72	20	7	72

Note: Gradient PCR program (start temperature 53°C, end temperature 48°C; 0.5°C decrease per cycle; 11 cycles from start to the end).

RNA was a popular assay in the 1980s [10, 11, 13, 15]. However, this technique is laborious and time consuming. With respect to polyacrylamide gel electrophoresis, migration patterns of RNAs derived for MDV and wild type virus may be difficult to distinguish.

An alternative approach is the use of multiplex RT-PCRs that yield a yes/no result [4, 5, 9]. This method is currently in use both for typing of influenza A and B viruses, subtyping of influenza A virus and for screening of reassortant live influenza vaccine virus strains. In brief, primers are selected that specifically amplify the genes encoding the internal proteins of the MDV and the surface glycoproteins HA and NA of the wild type virus. By optimizing the primers and PCR conditions, multiple primer sets can be mixed and more than one PCR reaction can be run in a single tube. The authors were able to determine the origin of all eight gene segments of reassortants in a total of three PCR reactions which allows for screening of numerous viruses in a relatively short period of time. However, using the multiplex PCRs no answer can be given about cleanliness of the reassortant virus. It cannot be ruled out that the reassortant virus is contaminated with another reassortant virus containing one or more wild type virus gene segments as these are not detected in their PCRs. The multiplex PCR definitely is attractive for initial screening, but needs additional PCRs or sequencing to rule out contamination with other reassortant viruses containing wild type gene segments.

One of the most reliable method is RT-PCR followed by digestion of PCR products with restriction enzymes (Restriction Fragment Length Polymorphism or RFLP) [2, 3, 7, 12, 14, 17]. RFLPs reveal differences in sequences of RNA segments as well as by different lengths of the restriction fragments produced by enzymatic digestion of a selected region of gene. Digested products are detected by ethidium bromide staining after agarose gel electrophoresis. With respect to RFLP, the use of restriction enzymes has several specific disadvantages, for instance, restriction enzymes may not always fully cut a PCR product, which complicates the determination of the origin of

Table 2. Primers used for screening of reassortants obtained from co-infections of currently circulating influenza A (H1N1) viruses with A/Leningrad/134/17/57 (H2N2) master donor virus

Gene segment	Parental virus	Nucleotide position[3]	Fragment length	Sense primer, F		Anti-sense primer, R		Program code[4]
				primer sequence, 5' to 3'	length, bp	primer sequence, 5' to 3'	length, bp	
PB2	WT[1]	330–1108	778	AAATGGACCAGTGGCAARTACTA	23	CATATCCCTCATGCACADTTAGC	23	4
	L17[2]	1068–1479	411	CAATCTTCAAACATTGAAAATAAGG	25	GCCCATTTTGCTGACTCTTAAC	22	4
PB1	WT	433–692	259	CAGCCTGCTGCCACAGCATTG	21	GTTAATGCTCTAATTAGGTAAC	22	4
	L17	1206–1816	610	TCTCCTAATAGATGGCACAGTC	22	AGTTTGGTCCTCCATCCGAAAT	22	4
PA	WT	1509–2036	527	GAGAAAGACCAATTTGTACGGC	22	GCCTGAACAATGAGGAGCAAC	21	4
	L17	798–1065	267	GCCTTTTCTGAAAACAACACCAA	23	GTCCTGCAGTTCTGCCAGTAAT	22	4
HA	WT	1–1110	1109	AGCAAAAGCAGGGGAAAATAA	21	CCACCCATCTACCATTCC	18	4
	L17	6–891	885	AAGCAGGGGTTATACCATAGACAAC-CAAAA	30	CCTTCTGTTTTCATGATCCCTGAAC-TACCT	30	4
NP	WT	1249–1502	253	TCTGCGGGCCAAATCAGCAC	20	GATCCTTCATTACTCATGTCAAAG	24	4
	L17	666–1121	455	GAG AGGTGAGAATGGGCGG	19	GAAAGTTTCCCCCTTGGGAT	20	4
NA	WT	427–643	216	AAGGTGCTCTATTAAAT	17	TATTTTAGTACAGCCAC	17	12
	L17	51–750	699	GCTCTGTYTCTCTCACCATT	20	CCATCAGTCATTACTACTG	19	12
M	WT	192–470	278	CTAAGGGGATTTTAGGATTTGTG	23	CACATATAAGGCCAAATGCTGA	22	4
	L17	455–994	539	TGAAGTGGCCTTGGGCCTGG	20	TTTTCAGACCGTGTTTAAAGAAG	23	4
NS	WT	441–711	270	GACCGGTTGGAGAATCTGAC	20	AGCCATCTTATTTCTTCAAACTTC	24	4
	L17	14–788	774	TGACAAAGACATAATGGATCCTA	23	ATTTGCTCAAAACTATTCTCTGTTA	25	4

Note: [1]Currently circulating epidemic (seasonal) influenza A (H1N1) viruses.
[2]A/Leningrad/134/17/57 (H2N2) master donor virus.
[3]Nucleotide position represents the nucleotide from the 5'-end of the positive sense genome.
[4]See program details in Table 1.

Table 3. Primers used for screening of reassortants obtained from co-infections of currently circulating influenza A (H3N2) viruses with A/Leningrad/134/17/57 (H2N2) master donor virus

Gene segment	Parental virus	Nucleotide position[3]	Fragment length	Sense primer, F		Anti-sense primer, R		Program code[4]
				primer sequence, 5' to 3'	length, bp	primer sequence, 5' to 3'	length, bp	
PB2	WT[1]	1477–2115	638	GGTGTGGATGAATACTCCAGTA	22	CTATAATGAGAAACCCTCTCAAG	23	4
	L17[2]	1273–2118	845	GGTGATCTGAATTTCGTTAATAGG	24	CAGAATGAGGAATCCTCTCAGA	22	4
PB1	WT	1755–2223	468	GAAGCTGTGGGATCAAACCCAA	22	TCCAGACTCGAAGTCAATTCTG	22	4
	L17	1206–2169	963	TCTCCTAATAGATGGCACAGTC	22	CACCATGCTGGAAATTCCAACT	22	4
PA	WT	970–1851	881	GGATGGAAAGAACCTTATATAGTC	24	TTTGGTCATGTCTTTCTCTTTAATC	25	4
	L17	914–1712	798	ACGAAGGAGAGGGAATACCAC	21	AAGAACATGGGCCTTGACACC	21	4
HA	WT	29–593	564	CATGAAGACCATCATTGCTTTGAGCTAC	28	GTCATTGTTTGGCATAGTCACGTTCAG	27	4
	L17	6–858	852	AAGCAGGGGTTATACCATAGACAAC-CAAAA	30	TTCGATATTTTGAATCCATACTCTGGT-GCA	30	4
NP	WT	135–1122	987	GAAGATGATTGATGGAATTGGGA	23	TGAMAGTTTCCCYCGMGGAGA	21	4
	L17	640–1331	691	CGTGGGATCAATGATCGGAAC	21	GAATGCTGCCATGATGGTTGGT	22	4
NA	WT	34–568	534	AAAGATAATAACRATTGGCTCTGTT	25	ACAACTTGAGCTGGACCATGCTATG	25	2
	L17	902–1334	432	TGGAAAGGCTCTAATAGGCCCGTTA	25	TCCACCATACTCTAGTCTCCTGTGG	25	2
M	WT	51–547	496	CGTATGTTCTCTCTATCGTTCCA	23	TYTTATTAATGGATTGGTTGTTGC	24	4
	L17	53–887	834	TACGTTCTCTCTATCGTCCCG	21	ACCGTGTTTAAAGAAGCGATAATT	24	4
NS	WT	86–478	392	ACAAGTTGTAGACCAAGAACTGA	23	GGTGAAAGCCCTTAGTAATACTAT	24	4
	L17	13–726	713	GTGACAAAGACATAATGGATCCT	23	TATTTCTTCGAACTTTTGACCTAAT	25	4

Note: [1]Currently circulating epidemic (seasonal) influenza A (H3N2) viruses.
[2]A/Leningrad/134/17/57 (H2N2) master donor virus.
[3]Nucleotide position represents the nucleotide from the 5'-end of the positive sense genome.
[4]See program details in Table 1.

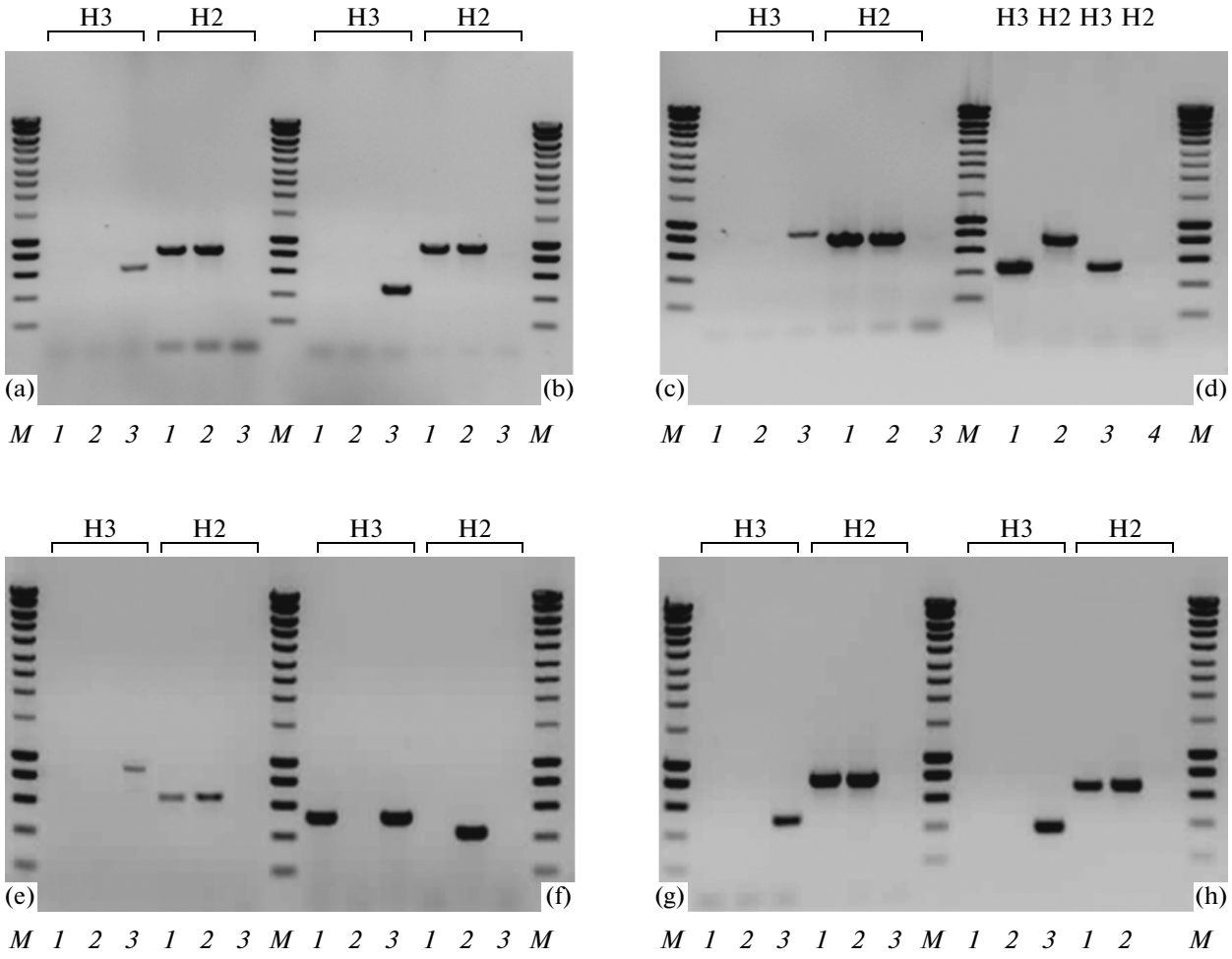

Fig. 1. PCR-identification of the genome composition of a reassortant virus obtained from co-infection of the A/Leningrad/134/17/57 (H2N2) master donor virus and the A/Brisbane/10/2007 (H3N2) seasonal influenza virus.

On the top of panels—virus specific primers: H3—specific primers to A (H3N2) seasonal influenza viruses; H2—specific primers to A/Leningrad/134/17/57 (H2N2) master donor virus. *On the bottom of panels*—tested viruses: *1*—reassortant virus; *2*—A/Leningrad/134/17/57 (H2N2) master donor virus; *3*—A/Brisbane/10/2007 (H3N2) seasonal influenza virus; *4*—A/Brisbane/59/2007 (H1N1) seasonal influenza virus; *M*—molecular weight marker; (a)—PB2 gene; (b)—PB1 gene; (c)—PA gene; (d)—HA gene; (e)—NP gene; (f)—NA gene; (g)—M gene; (h)—NS gene.

gene segments of reassortants. Incomplete digestion may result from insufficient enzyme activity or could indicate that the virus is not clean.

The aim of this study was to design a PCR method using gene segment and virus (sub) type specific primers allowing for rapid and unequivocal screening of reassortant influenza A and influenza B viruses to be used in seasonal and pandemic LAIV.

Seasonal influenza A (H1N1 and H3N2) virus specific primers. Virus subtype specific primer sets were designed for each of the gene segments of wild type seasonal influenza A H1N1 virus and the A/Leningrad/134/17/57 (H2N2) MDV (Tables 2, 3). H1N1 or H3N2 specific primers only yielded PCR products for gene segments derived from H1N1 or H3N2 virus respectively, while MDV specific primers only yielded PCR products for MDV derived gene segments. Moreover, the PCR product obtained with wild type virus specific primers differed in size from the PCR product obtained with MDV specific primers. Thus, the yes/no result and the difference in size make it easy to discriminate the PCR products on gel and to determine the origin of each of the gene segments in a reassortant virus.

An example is shown in Fig. 1. As can be seen in this figure, reassortant possessed HA and NA from A/Brisbane/10/2007 (H3N2) wild type virus, which was in use for vaccine production in the 2009–2010 influenza season. Vaccine candidate virus yielded PCR products for HA (Fig. 1d) and NA (Fig. 1f) using

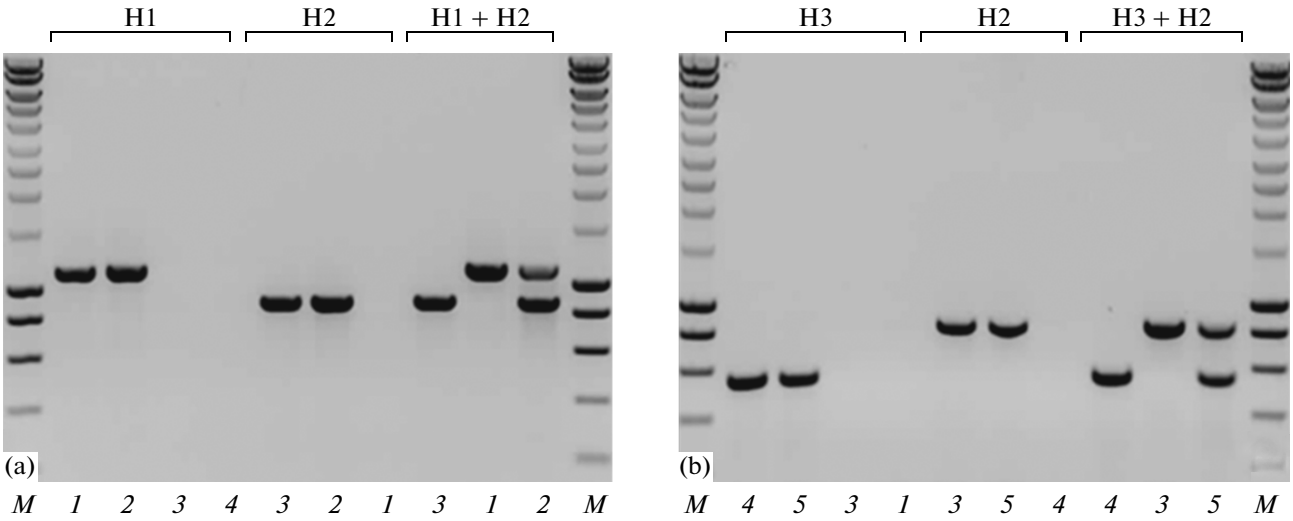

Fig. 2. Mixed PCR analysis of haemagglutinin gene segment belonging to A (H1N1), A (H3N2) or A (H2N2) influenza virus. *On the top of panels*—tested viruses: H1—A/Brisbane/59/2007 (H1N1) seasonal influenza virus; H2—A/Leningrad/134/17/57 (H2N2) master donor virus; H1 + H2—mixture of A/Brisbane/59/2007 (H1N1) with A/Leningrad/134/17/57 (H2N2) viruses; H3—A/Brisbane/10/2007 (H3N2) seasonal influenza virus; H3 + H2—mixture of A/Brisbane/10/2007 (H3N2) with A/Leningrad/134/17/57 (H2N2) viruses. *On the bottom of panels*—virus specific primers to influenza virus haemagglutinin: *1*—specific primers to A (H1N1) seasonal influenza viruses; *2*—mixture of specific primers to A (H1N1) seasonal influenza viruses with specific primers to A/Leningrad/134/17/57 (H2N2) master donor virus; *3*—specific primers to A/Leningrad/134/17/57 (H2N2) master donor virus; *4*—specific primers to A (H3N2) seasonal influenza viruses; *5*—mixture of specific primers to A (H3N2) seasonal influenza viruses with specific primers to A/Leningrad/134/17/57 (H2N2) master donor virus; *M*—molecular weight marker. Left panel (a)—distinguishing haemagglutinin gene segment of A (H1N1) seasonal influenza virus from those of A/Leningrad/134/17/57 (H2N2) master donor virus. Right panel (b)—distinguishing haemagglutinin gene segment of A (H3N2) seasonal influenza virus from those of A/Leningrad/134/17/57 (H2N2) master donor virus.

H3N2 specific primers but not when MDV specific primers were used). Contrary, the reassortant H3N2 virus yielded PCR products using MDV specific primers for PB2 (Figure 1a), PB1 (Fig. 1b), PA (Fig. 1c), NP (Fig. 1e), M (Fig. 1g) and NS (Fig. 1h) gene segments but no PCR products were obtained using H3N2 specific primers, clearly demonstrating that the six gene segments encoding the internal viral proteins all originated from MDV.

Mixed RT-PCR. For the determination of the origin of the HA and NA encoding gene segments, so-called mixed PCRs were developed whereby in a single reaction MDV and wild type seasonal influenza A (H1N1 or H3N2) specific primers were used. The primers were selected in different regions of the gene segments resulting in different sizes of PCR products obtained for MDV and wild type virus.

An example is shown in Fig. 2. As can be seen in the left panel (Fig. 2a), H1N1 virus yields a PCR product with H1 specific primers and a mixture of PCR product with H1 and H2 (MDV) specific primers, but not with H2 or H3 specific primers alone. Contrary, MDV yields a PCR product using H2 specific primers and a mixture with H1 and H2 specific primers, but not with H1 specific primers alone. Since the size of the PCR product obtained with the H1 specific primers differs from that obtained with H2 specific primers, the mixed H1/H2 PCR can be used to easily determine the virus subtype in a single PCR reaction. In case of a mixture of two different subtypes, two clearly separated PCR products are obtained in this mixed PCR. Thus, in the event that the serum used during selective passages following co-infection is not completely blocking viruses containing the haemagglutinin of MDV, this will be clearly visible in the mix PCR. Analogous to the H1/H2 mixed PCR, a H3/H2 mixed PCR was developed to distinguish wild type and reassortant H3 viruses from H2 viruses (right panel, Fig. 2b).

In addition to the mixed PCR for determination of the origin of HA, a mixed PCR was developed to distinguish neuraminidase N1 from N2. As can be seen in Fig. 3, A/Brisbane/59/2007 (H1N1) virus, yields a PCR product with N1 specific primers or a mixture with N1 and N2 specific primers but not with N2 (MDV) specific primers. The opposite is found for MDV. As with the H1/H2 mixed PCR, a mixture of two viruses will easily be detected as two bands of different size will appear in gel electrophoresis.

Screening of reassortant viruses in this way yielded unambiguous results. Indeed, for each individual gene

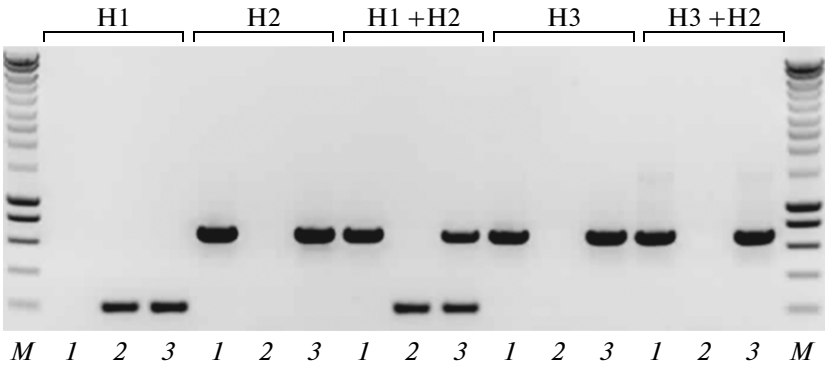

Fig. 3. Mixed PCR analysis of neuraminidase gene segment belonging to A (H1N1) or A (H2N2) influenza virus.
On the top of panel—tested viruses: H1—A/Brisbane/59/2007 (H1N1) seasonal influenza virus; H2—A/Leningrad/134/17/57 (H2N2) master donor virus; H3—A/Brisbane/10/2007 (H3N2) seasonal influenza virus; H1 + H2—mixture of A/Brisbane/59/2007 (H1N1) with A/Leningrad/134/17/57 (H2N2) viruses; + H2—mixture of A/Brisbane/10/2007 (H3N2) with A/Leningrad/134/17/57 (H2N2) viruses. *On the bottom of panels*—virus specific primers to influenza virus neuraminidase: *1*—specific primers to A/Leningrad/134/17/57 (H2N2) master donor virus; *2*—specific primers to neuraminidase of A (H1N1) seasonal influenza viruses; *3*—mixture of specific primers to A/Leningrad/134/17/57 (H2N2) master donor virus and A (H1N1) seasonal influenza viruses; *M*—molecular weight marker.

segment of both the MDV and the wild type parental viruses, PCR products of different sizes were obtained with MDV and wild type virus specific primer sets, respectively. Using the primers the other way around (MDV with wild type virus specific primers and wild type virus with MDV specific primers) no PCR products were obtained. When reassortant viruses are analyzed in this way, the genome composition will immediately be clear and, importantly, will demonstrate whether or not the virus is of a single genotype. In case of a mixture of two or more genetically different viruses this will become clear by the appearance of two different sized PCR products for one or more gene segments. This demonstrates that the mixed PCR assay is very specific and may also be used for typing of influenza A and B isolates from influenza patients and from individuals vaccinated with LAIV.

Seasonal influenza B virus specific primers. Regions of naturally occurring sequence diversity between currently circulating wild type influenza B viruses and the B/USSR/60/69 MDV were identified to design gene segment specific primer sets analogous to wild type influenza A viruses and A/Leningrad/134/17/57 (H2N2) MDV (Table 4).

Pandemic and prepandemic influenza A (H1N1 and H5N1) virus specific primers. In 2009, a novel H1N1 virus appeared that rapidly spread around the globe resulting in the first influenza pandemic of 21st century. This H1N1 virus (of which A/California/07/09 (H1N1) is an example) is quite different from the H1N1 virus that circulated previously. Therefore, new primers were designed that allowed for discrimination of gene segments of this novel H1N1 virus from those of MDV (Table 5).

Today both circulating H1N1 California-like influenza viruses of swine origin and highly pathogenic A (H5N1) avian influenza viruses still confer a potential influenza pandemic risk. The use of highly pathogenic influenza viruses with pandemic potential in the generation of vaccine viruses to be used in LAIV is undesired from an operator safety point of view. In these cases alternatives are to be sought for donation of the proper surface glycoproteins of A (H5N1) avian influenza virus in an MDV backbone. The use of reassortant viruses based on A/PR/8/34 (H1N1) could be an alternative to wild type virus. A/PR/8/34 (H1N1) is routinely used for generating high yield reassortant influenza A viruses for inactivated influenza vaccines including H5N1. In this case, the HA of the H5N1 virus was first modified by removal of the multibasic cleavage site to make haemagglutinin apathogenic. Than modified HA together with NA were put in a background of A/PR/8/34 (H1N1) using reverse genetics [16]. These H5N1–PR8 based reassortant viruses produced by NIBSC (UK) and CDC (USA) could well be used in co-infections with cold-adapted MDV to generate new reassortant viruses. Replacement of PR8 internal genes in the genome of H5N1–PR8 based reassortants with appropriate genes of MDV will lead to the generation of a prepandemic LAIV candidate. Also for these situations, primers were designed that enabled discrimination between six internal gene segments derived from MDV or

Table 4. Primers used for screening of reassortants obtained from co-infections of currently circulating influenza B viruses with B/USSR/60/69 master donor virus

Gene segment	Parental virus	Nucleotide position[3]	Fragment length	Sense primer, F		Anti-sense primer, R		Program code[4]
				primer sequence, 5' to 3'	length, bp	primer sequence, 5' to 3'	length, bp	
PB2	WT[1]	987–1910	923	AGACAAAGACAAAGATTTGGAC	22	CCTTAATTTTGGTGGTGAGAAACAA	25	7
	B60[2]	805–1030	225	TGATTGTAGCTTGTAGGAAAATAG	24	GATATTCTCTTTAGTTCAAGCCTC	24	7
PB1	WT	1409–1830	421	ACGACTTTTACCGAACATGTAAAT	24	YAAGTTTCTCAAATTGTAAATGTTG	25	8
	B60	525–1171	646	AGATATCATTGATTCATTGGACAAA	25	CTTCATTATATCTTTCTAATGGTATAT	27	6
PA	WT	694–1106	412	GTYCTCATAGGRGAAGAAGATG	22	GGTTTTCTGTAAYTCGTTACTC	22	6
	B60	1920–2237	317	AAAGCACTAAGAGTAATATTCACC	24	TACCAAATTG AGCGCTATGCTC	22	9
HA	WT	194–484	290	CTYATTTTGCAAATCTCAAAGGAACAAR	28	TGTAGGGTCCTCCTGGTGCHT	21	1
	B60	504–823	319	TTGCCCTAACGTTACCAATGGGAAA	25	CAACAACAATTCTGCCGCTTTGTTT	25	11
NP	WT	995–1209	214	GAYCTAACCCTGCTTGCTCGT	21	AATTGYGATTTRTCYTTTGCATCA	24	9
	B60	1164–1784	620	TTTCCATATTAAGAATGGGAGACA	24	AACGTACTGAAACAGTCACAGC	22	5
NA	WT	470–841	371	GGGGGATACTACAATGGAACAAGAG	25	TTTTATTATTCGGCCCTCTCGAATC	25	3
	B60	871–1109	238	TACTGAAGAATGCACATGCGGGTTC	25	CAAATCCTCCTTTGATGCCTCCAAG	25	3
M	WT	486–1137	651	AGCATCACATTCACACAGGGCT	22	ACGGGGCTGCAACTTATTTGA	21	5
	B60	920–374	546	AAGGCCACGAAAGCTCAGCAT	21	GTTTATTGTCTCTTTATTTGGATT	24	6
NS	WT	764–1032	268	AACTCACTCTTCGAGCGTCTTA	22	CCTTCATTTCATACAATGTTTCTAAT	26	10
	B60	369–804	435	CAACTAGCAACTGTCCAAACTG	22	GGCTTTGAATGTCCTTCATCAAA	23	7

Note: [1]Currently circulating wild type influenza B viruses (Victoria and Yamagata lineages).
[2]B/USSR/60/69 master donor virus.
[3]Nucleotide position represents the nucleotide from the 5'-end of the positive sense genome.
[4]See program details in Table 1.

Table 5. Primers used for screening of reassortants obtained from co-infections of pandemic A/California/07/2009 (H1N1)—like viruses with A/Leningrad/134/17/57 (H2N2) master donor virus

Gene segment	Parental virus	Nucleotide position[3]	Fragment length	Sense primer, F		Anti-sense primer, R		Program code[4]
				primer sequence, 5' to 3'	length, bp	primer sequence, 5' to 3'	length, bp	
PB2	WT[1]	1414–1781	367	GAGATGTCGCTGAGAGGGA	19	CCACTGTACCGGCTTCTGG	19	4
	L17[2]	1068–1479	411	CAATCTTCAAACATTGAAAATAAGG	25	GCCCATTTTGCTGACTCTTAAC	22	4
PB1	WT	301–588	287	GGAATATTTGAGAATTCATGCC	22	GGTCATGTTGTCTCTTACTCTC	22	4
	L17	1206–2169	963	TCTCCTAATAGATGGCACAGTC	22	CACCATGCTGGAAATTCCAACT	22	4
PA	WT	151–832	651	TTCCATTTCATCGACGAACGG	21	GATGGCAAAGAGGCCCATCA	20	4
	L17	798–1065	267	GCCTTTTCTGAAAACAACACCAA	23	GTCCTGCAGTTCTGCCAGTAAT	22	4
HA	WT	245–842	597	CAGAGTGTGAATCACTCTCCA	21	CCAGATCCAGCATTTCTTTC	21	4
	L17	6–891	885	AAGCAGGGGTTATACCATAGACAAC-CAAAA	30	CCTTCTGTTTTCATGATCCCTGAAC-TACCT	30	4
NP	WT	35–662	627	AAATGGAGACTGGTGGGGAG	20	CTTTCATAAGCAACCCTTGTCC	22	4
	L17	640–1331	691	CGTGGGATCAATGATCGGAAC	21	GAATGCTGCCATGATGGTTGGT	22	4
NA	WT	770–1114	334	GAATAGAAAAGGGAAAGATAGTC	23	CAAAACCGTTTCTTGAACTAATG	23	12
	L17	902–1334	432	TGGAAAGGCTCTAATAGGCCCGTTA	25	TCCACCATACTCTAGTCTCCTGTGG	25	2
M	WT	325–789	464	TTCCATGGGGCCAAGGAGG	19	CCAATGATATTTGCTGCAATGAC	23	4
	L17	455–994	539	TGAAGTGGCCTTGGGCCTGG	20	TTTTCAGACCGTGTTTAAAGAAG	23	4
NS	WT	334–758	424	GTAGGCCCTCTTTGCGTGC	19	GTTCGAAACTATTCTCTGTCGCT	23	4
	L17	13–726	713	GTGACAAAGACATAATGGATCCT	23	TATTTCTTCGAACTTTTGACCTAAT	25	4

Note: [1]Currently circulating wild type A/California/07/09 (H1N1)-like viruses.

[2]A/Leningrad/134/17/57 (H2N2) master donor virus.

[3]Nucleotide position represents the nucleotide from the 5'-end of the positive sense genome.

[4]See program details in Table 1.

Table 6. Primers used for screening of reassortants against highly pathogenic avian influenza viruses obtained from co-infections of A/PR/8/34 (H1N1)-based A (H5N1) reassortant viruses for inactivated vaccine with A/Leningrad/134/17/57 (H2N2) master donor virus

Gene segment	Parental virus	Nucleotide position[3]	Fragment length	Sense primer, F		Anti-sense primer, R		Program code[4]
				primer sequence, 5' to 3'	length, bp	primer sequence, 5' to 3'	length, bp	
PB2	WT1	248–598	350	ATGAATGATGCCGGATCAGAC	21	TGTTCCTAGCAGCAATAATCAAG	23	4
	L17[2]	1068–1479	411	TTAATAGGGCAAATCAGCGATTG	24	GAATTGTTAGTCTCTTAGTGGTC	23	4
PB1	WT[1]	1407–1910	503	CGGAGTCGACAGGTTTTATC	20	TTCAGTGGGTTGCATAAACGC	21	4
	L17	1206–1816	610	TCTCCTAATAGATGGCACAGTC	22	AGTTTG GTCCTCCATCCGAAAT	22	4
PA	WT[1]	836–1241	405	CGAATGGGCCTCCCTGTTC	19	CAACTTGCAAGCGACCTCAAT	21	4
	L17	798–1065	267	GCCTTTTCTGAAAACAACACCAA	23	GTCCTGCAGTTCTGCCAGTAAT	22	4
HA	WT	319–901	582	AAGATCAATCCAGCCAATGACC	22	TGTTGCAGTTACCATATTCCAAC	23	4
	L17	6–891	885	AAGCAGGGGTTATACCATAGACAAC-CAAAA	30	CCTTCTGTTTTCATGATCCCTGAAC-TACCT	30	4
NP	WT[1]	1085–1352	267	TCAAAGGGACGAAGGTGCTC	20	CTCCCCTCTGTATTCCCAT	19	4
	L17	666–1121	455	GAGAGGTGAGAATGGGCGG	19	GAAAGTTTCCCCCTTGGGAT	20	4
NA	WT[4]	425–1186	761	GTTGTCCTGTGGGTGAGGC	19	CTGTCAGTTCTGGATGCTGG	20	12
	L17	51–750	699	GCTCTGTYTCTCTCACCATT	20	CCATCAGTCATTACTACTG	19	12
M	WT[1]	503–814	311	GCTAGTCAGGCTAGACAAATG	21	CAGTCCGTATTTAAAGCGACGG	20	4
	L17	455–994	539	TACGTTCTCTCTATCGTCCCG	21	ACCGTGTTTAAAGAAGCGATAATT	24	4
NS	WT[1]	212–784	572	GACATCGAGACAGCCACAC	19	CCTTAGCAATATTAGAGTCTCC	22	4
	L17	14–788	774	GGGACTGGTTCATGCTAATGC	21	CTTGTTCCACAAATAGTAGC	23	4

Note: [1]A/PR/8/34 (H1N1) influenza virus—donor of internal genes for inactivated influenza vaccine strains.
[2]A/Leningrad/134/17/57 (H2N2) master donor virus.
[3]HA and NA of current A (H5N1) avian influenza virus.
[4]Nucleotide position represents the nucleotide from the 5'-end of the positive sense genome.
[5]See program details in Table 1.

A/PR/8/34 (H1N1) virus, and HA and NA derived from MDV and H5N1 virus, respectively (Table 6).

In conclusion, RT-PCRs were developed that unambiguously demonstrate the genome composition of A and B reassortant influenza viruses. These RT-PCRs allow for the clear and rapid selection of both influenza A and influenza B vaccine viruses (6 : 2 reassortant viruses) to be used in LAIV production.

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