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Eight-plasmid system for rapid generation of influenza virus vaccines

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Abstract

The antigenic variation of influenza A virus hemagglutinin (HA) and neuraminidase (NA) glycoproteins requires frequent changes in vaccine formulation. The classical method of creating influenza virus seed strains for vaccine production is to generate 6 + 2 reassortants that contain six genes from a high-yield virus, such as A/PR/8/34 (H1N1) and the *HA* and *NA* genes of the circulating strains. The techniques currently used are time-consuming because of the selection process required to isolate the reassortant virus. We generated the high-yield virus A/PR/8/34 (H1N1) entirely from eight plasmids. Its growth phenotype in embryonated chicken eggs was equivalent to that of the wild-type virus. By using this DNA-based cotransfection technique, we generated 6 + 2 reassortants that had the antigenic determinants of the influenza virus strains A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2), A/teal/HK/W312 (H6N1), and A/quail/HK/G1/97 (H9N2). Our findings demonstrate that the eight-plasmid system allows the rapid and reproducible generation of reassortant influenza A viruses for use in the manufacture of vaccines.

Keywords: Influenza; Vaccines; Reverse genetics

1. Introduction

During the 20th century, influenza A viruses of the H1N1, H2N2, and H3N2 antigenic subtypes have caused epidemics of respiratory disease in humans. In 1997, H5N1 viruses of avian origin caused human illness and death in China [1,2]. In 1998 and 1999, H9N2 viruses circulating in poultry caused disease in humans [3]. Sequence analysis of the *HA* gene of the human isolate A/HK/1073/99 (H9N2) revealed the virus to be closely related to a virus isolated from quail, A/quail/HK/G1/97 (H9N2) [4]. These influenza outbreaks raised concerns about newly emerging influenza A viruses and the possibility of a new pandemic [5]. At the time of the outbreaks, no vaccines were available for protection against the H5 subtype.

The licensed influenza vaccines in current use are inactivated virus vaccines created by growing virus in embryonated chicken eggs and subsequently purifying and inactivating them by chemical means. Each year, the World Health Organization selects subtypes that are representative of strains currently circulating in humans. The efficacy of vaccines requires that the selected vaccine strains be sufficiently closely related to the circulating strains to ensure the induction of effective neutralizing antibodies. However, not all viruses that are closely related are suitable for vaccine production; some grow poorly in eggs. Therefore, the current practice is to generate a high-growth reassortant that combines the high virus yield of the laboratory strain A/PR/8/34 (H1N1) with expression of the glycoproteins of the currently circulating strain [6].

Coinfection with two influenza viruses containing eight segments can theoretically result in the generation of $2^8 - 2 = 254$ different progeny viruses. The selection procedure required to obtain the desired reassortant virus and to verify its gene constellation is cumbersome and time-consuming [7]. Although the reverse genetics method in which cells are transfected with in vitro-generated ribonucleoproteins, reduces the possible number of progeny viruses, a more efficient selection method is needed [8]. Plasmid-driven synthesis of viral RNA and proteins allows the recovery of infectious influenza virus without the need for helper virus infection [9–11]. Therefore, we investigated whether the eight-plasmid system which we established [11] allows the generation of 6+2 reassortant viruses. Here, we demonstrate the utility of this approach for the generation of high-yield reassortants. Our findings suggest that vaccine manufacturers can apply this DNA transfection method to cell lines

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Table 1
Generation of 6 + 2 reassortant influenza A viruses

HA/NA-subtype	Parent virus	HA plasmid	NA plasmid	Recombinant virus[a]
H1N1	A/New Caledonia/20/99 (H1N1)	pHW454-HA	pHW456-NA	rgPR8-H1N1
H3N2	A/Panama/2007/99 (H3N2)	pHW444-HA	pHW446-NA	rgPR8-H3N2
H5N1	A/Goose/HK/437-4/99 (H5N1)	pHW251-HA	pHW246-NA	rgPR8-H5ΔN1[b]
H6N1	A/teal/HK/W312/97 (H6N1)	pHW244-HA	pHW246-NA	rgPR8-H6N1
H9N2	A/quail/HK/G1/97 (H9N2)	pHW409-HA	pHW422-NA	rgPR8-H9N2

[a] Viruses were generated by transfecting cocultured 293T–MDCK cells with six plasmids carrying the six internal genes of the virus strain A/PR/8/34 (H1N1) and two plasmids encoding the desired HA and NA subtypes.
[b] The N1 neuraminidase was derived from A/teal/HK/W312/97 (H6N1).

which are approved for vaccine production for rapid and reproducible generation of influenza virus vaccines.

2. Materials and methods

2.1. Virus strains

The influenza A viruses used in this study (Table 1) were obtained from the repository of St. Jude Children's Research Hospital and were propagated in embryonated chicken eggs.

2.2. RT-PCR and construction of plasmids

RT-PCR was performed with segment-specific primers as described elsewhere [12]. Briefly, RNA was isolated by using the RNeasy kit (Qiagen). RNA was transcribed to cDNA by using Uni12-primer (AGC AAA AGC AGG) and the cDNA was then amplified by using segment-specific primers. The *HA*, *NP*, *NA*, *M*, and *NS* genes of A/PR/8/34 (H1N1) (PR8 virus) were cloned by digesting the PCR fragments with *Bsm*BI or *Bsa*I and ligating them into the cloning vector pHW2000. The *P* genes were cloned by isolating two (PB2, PA) or three (PB1) fragments, digesting them, and ligating them into pHW2000-*Bsm*BI. To ensure that the genes were free of unwanted mutations, the inserted viral cDNAs were sequenced. The eight plasmids containing the full-length cDNA of PR8 virus were designated pHW191-PB2, pHW192-PB1, pHW193-PA, pHW194-HA, pHW195-NP, pHW196-NA, pHW197-M, and pHW198-NS. PCR with the primer pair Bm-HA1 (TAT TCG TCT CAG GGA GCA AAA GCA GGG G) and Bm-NS-890R (ATA TCG TCT CGT ATT AGT AGA AAC AAG GGT GTT TT) was used to clone *HA* genes of several subtypes (nucleotides representing the influenza A virus non-coding regions are underlined). Those primers were also used to characterize the recombinant viruses by RT-PCR. The plasmid pHW251-HA encoding a deletion mutant of the A/Goose/HK/437-4/99 (H5N1) hemagglutinin was derived by PCR amplification of two fragments of the plasmid pHW250-HA encoding the full-length H5. The fragments were digested with *Bsm*BI and inserted into pHW2000-*Bsm*BI. For cloning of pHW409-HA, the H9-PCR fragment was first inserted into the pCR2.1 vector (invitrogen); the resultant plasmid containing the *H9* gene was used to subclone the *H9* gene into pHW2000-*Bsm*BI. The construction of the plasmids pHW244-HA and pHW246-NA representing the *HA* and *NA* genes from A/teal/HK/W312 (H6N1) has been described elsewhere [11]. The Center for Biotechnology at St. Jude Children's Research Hospital determined the sequence of template DNA by using Rhodamine or dRhodamine dye-terminator cycle sequencing ready reaction kits with AmpliTaq® DNA polymerase FS (Perkin-Elmer Applied Biosystems Inc., Foster City, CA) and synthetic oligonucleotides. Samples were separated by electrophoresis and analyzed on PE/ABI model 373, model 373 Stretch, or model 377 DNA sequencers.

2.3. Generation of recombinant viruses

Recombinant viruses were generated by DNA transfection as described previously [11]. Briefly, the day before transfection 293T and MDCK cells were trypsinized, $(0.2–1) \times 10^6$ of each cell line were used for the transfection experiments. Different dilutions ($1{:}10^3$–$1{:}10^5$) of virus stocks containing 320–5120 hemagglutination units (HAU) per ml were used to infect the allantoic cavity of 10-day-old embryonated chicken eggs. The data in Table 2 represent results from two or three experiments.

After 48 h, the allantoic fluid was harvested for analysis. H5N1, H6N1, and H9N2 viruses were grown in BL3 facilities at St. Jude Children's Research Hospital.

2.4. Hemagglutination (HA) and hemagglutination inhibition (HI) assays

Fifty microliters of 0.5% chicken red blood cell suspension in PBS was added to 50 μl of two-fold dilutions of virus in phosphate buffered saline (PBS), and the mixture was incubated at room temperature for 30 min. The HA titre was calculated as the reciprocal value of the highest virus dilution that caused complete hemagglutination.

For HI assays, 50 μl of receptor destroying enzyme treated antiserum was titrated, and 25 μl of an equivalent amount of virus (four hemagglutinating doses) was added to each well. After incubation at room temperature for 30 min, 50 μl of a

Table 2
Growth of 6 + 2 reassortant influenza A viruses

Virus	HA titre[a]										
	1	2	3	4	5	6	7	8	9	10	Mean
wtPR8	10240	5120	10240	5120	10240	Dead	10240	10240	5120	10240	8533
rgPR8	10240	20480	320	10240	5120	640	5120	5120	2560	5120	6496
rgPR8-H1N1	2560	1280	1280	2560	2560	640	2560	5120	1280	1280	2112
rgPR8-H3N2	1280	1280	1280	1280	640	2560	2560	2560	1280	640	1536
rgPR8-H5ΔN1	160	320	160	160	320	160	160	320	320	160	224
rgPR8-H6N1	2560	5120	2560	1280	1280	1280	1280	1280	1280	2560	2048
rgPR8-H9N2	5120	5120	10240	5120	5120	5120	5120	10240	5120	5120	6144

[a] The HA titre of the allantoic fluid was determined 48 h after infection of 10-day-old embryonated chicken eggs. Assays were performed with 0.5% chicken red blood cells. Values represent the titres after infection of 10 different eggs.

0.5% suspension of chicken red blood cells was added. The HI titre was determined after 30 min as the reciprocal of the serum dilution that inhibited hemagglutination.

3. Results

3.1. Generation of A/PR/8/34 (H1N1) from eight plasmids

The influenza A virus A/PR/8/34 (H1N1) is well adapted to growth in embryonated chicken eggs and is currently used as the master strain for the production of inactivated vaccines. To generate A/PR/8/34 (H1N1) virus from plasmids, we amplified the eight viral RNA segments by RT-PCR and cloned the fragments into the plasmid pHW2000 [11]. Recombinant viruses were generated by transfection of cocultured 293T–MDCK cells with the resultant eight plasmids (pHW191-PB2, pHW192-PB1, pHW193-PA, pHW194-HA, pHW195-NP, pHW196-NA, pHW197-M, and pHW198-NS). The yield of virus recovered after 72 h was determined to be 2×10^6 pfu/ml by titration of the cell culture supernatant in MDCK cells. The supernatant of cocultured Vero-MDCK cells contained 1×10^4 pfu/ml.

To compare the growth of the wild-type virus (wtPR8) with that of the recombinant virus (rgPR8) generated by reverse genetics, we infected embryonated chicken eggs with wild-type or recombinant virus. The allantoic fluid of 10 infected eggs was harvested 48 h after infection. The virus yield was determined by HA assay. Although the HA titres differed among individual eggs, both viruses had HA titres between 5120 and 10,240 in most of the eggs and were therefore high-yielding isolates (Table 2). These results show that the A/PR/8/34 (H1N1) rgPR8 virus is generated efficiently and reliably from eight plasmids and that the plasmid-derived recombinant virus has the same high-yield phenotype as the wild-type virus.

3.2. Generation of the H3N2 and H1N1 6 + 2 reassortants recommended for human vaccine strains

The A/Panama/2007/99 (H3N2) and A/New Caledonia/20/99 (H1N1) virus strains were recommended by World Health Organization for use in influenza vaccine in the year 2001/2002. To test the utility of the eight-plasmid system to generate reassortants representing these strains, we cotransfected 293T–MDCK cells with plasmids encoding the glycoproteins of A/Panama/2007/99 (H3N2) or A/New Caledonia/20/99 (H1N1) and with the six plasmids encoding the internal genes of A/PR/8/34 (Fig. 1). We found the HA titres in the majority of infected eggs to be 1280–2560 (Table 2). The recombinant rgPR8-H1N1 and rgPR8-H3N2 viruses were shown by HI assay (Table 3) to be antigenically identical to the parental viruses A/Panama/2007/99 (H3N2) and A/New Caledonia/20/99 (H1N1).

3.3. Generation of 6 + 2 reassortants from viruses circulating in Southeast China

The pathogenic H5N1 viruses isolated in 1997 in Hong Kong were hypothesized to have been generated by reassort-

Table 3
Antigenic characterization of wild-type and recombinant viruses by hemagglutination inhibition (HI) assay

Virus	Antiserum	
	Specific[a]	α-H7[b]
wtPR8	2560	<[c]
rgPR8	640	<
A/New Caledonia/20/99 (H1N1)	>5120	80
rgPR8-H1N1	>5120	160
A/Panama/2007/99 (H3N2)	>5120	<
rgPR8-H3N2	>5120	<
A/Goose/HK/437-4/99 (H5N1)	2560	<
rgPR8-H5ΔN1	>5120	<
A/teal/HK/W312/97 (H6N1)	320	<
rgPR8-H6N1	80	<
A/quail/HK/G1/97 (H9N2)	1280	<
rgPR8-H9N2	1280	<
A/Eq/Prague	<	640

[a] Specific antiserum against wild-type virus was used for each subtype.
[b] A/equine/Prague/1/56 (H7N7)-specific antiserum was used as a control.
[c] <: No detectable inhibition of hemagglutination.

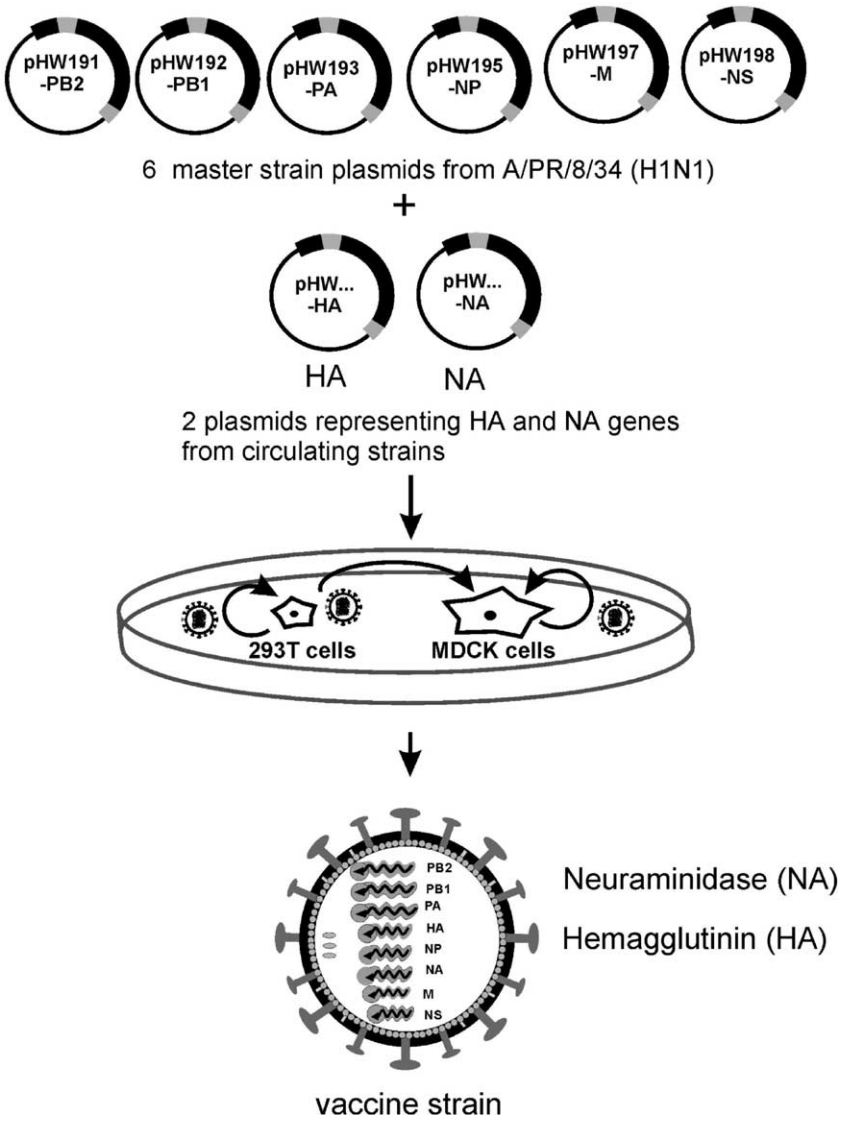

Fig. 1. Eight-plasmid system for rapid generation of reassortant influenza A virus. To test the utility of the eight-plasmid system for generation of reassortant viruses, 293T–MDCK cells were cotransfected with six plasmids representing the A/PR/8/34 (H1N1) master strain and two plasmids representing the desired HA and/or NA subtypes. Influenza viruses were generated within 2–3 days after transfection. Because the viruses were derived entirely from DNA, no selection system was needed to isolate the desired reassortant. The rescued viruses can be used as seed viruses for growth in embryonated chicken eggs.

ment of a Goose-H5 precursor virus [13]. The high homology of six segments of A/quail/HK/G1/97 (H9N2) and seven segments of A/teal/HK/W312/97 (H6N1) to those of the H5N1 viruses suggested that the H5N1 viruses were derived from H9N2 or H6N1 viruses circulating in Asia [4,14,15]. The close relationship of those viruses to the pathogenic H5N1 viruses which caused disease in humans indicates that those viruses could be transmitted to mammalian species. Indeed, H9N2 viruses closely related to the G1 lineage have been isolated from humans [3,16].

To evaluate whether 6 + 2 reassortants could be derived from currently circulating H6N1 and H9N2 subtypes, we cotransfected 293T–MDCK cells with plasmids containing the hemagglutinin and neuraminidase genes from A/teal/HK/W312/97 (H6N1) or A/quail/HK/G1/97 (H9N2) and with the six plasmids representing the internal genes of the PR8 virus. The HA titres of the reassortants were 5120–10,240 for rgPR8-H9N2 and 1280–5120 for rgPR8-H6N1 (Table 2). Antigenic analysis (Table 3) confirmed that the rgPR8-H6N1 and rgPR8-H9N2 viruses were of the same subtype as A/teal/HK/W312/97 (H6N1) and A/quail/HK/G1/97 (H9N2).

Preparation of high-growth reassortants for the production of H5N1 vaccine strains is difficult, because the infected chicken embryos die within 1 day after infection. The high lethality of H5N1 strains in chickens is associated with the basic amino acids of the connecting peptide between the HA1 and HA2 subunits. Therefore, we constructed a plasmid in which the region encoding the connecting peptide (PQ*RERRRKKR* ↓ G) of the H5

from A/Goose/HK/437-4/99 (H5N1) was replaced with a sequence found in the H6 of A/teal/HK/W312 (H6N1) (PQ*IETR* ↓ G). This plasmid and the plasmid encoding N1 neuraminidase from A/teal/HK/W312/97 (H6N1) (closely related to the neuraminidase of the pathogenic H5N1 viruses from 1997) together with the six PR8-plasmids were co-transfected into 293T–MDCK cells resulting in the generation of rgPR8-H5ΔN1 virus. This reassortant virus yielded an HA titre of 160–320, 48 h after infection (Table 2).

3.4. Characterization of the reassortant viruses by RT-PCR

Although controls were included in the experiments and no wild-type viruses were used during the DNA transfection experiments to minimize the possibility of laboratory contaminations, RT-PCR was performed to confirm that the recovered influenza viruses were reassortants with the PR8 backbone. The *NS* and *HA* genes of the 6 + 2 reassortants were amplified by RT-PCR with the primer pair Bm-HA1 and Bm-NS-890R, which were previously shown to amplify these genes [12]. Partial sequencing of the PCR products with segment-specific primers confirmed that the *HA* genes were derived from the designated subtypes and that all of the *NS* genes were derived from PR8 virus. These results show that reassortant influenza A viruses of the H1N1, H3N2, H5N1, H6N1, and H9N2 subtypes can be generated rapidly and reproducibly.

4. Discussion

The eight-plasmid virus generation system that uses the human RNA polymerase-I promoter requires the use of cell lines derived from humans or monkeys because of the species specificity of pol I−mediated transcription [17]. In this study, we used 293T cells for virus generation because those cells have high transfection efficiency resulting in high virus yield. To ensure the safety of a vaccine, cell lines approved for influenza virus vaccines, such as Vero monkey kidney cells can be employed for primary virus generation by DNA transfection. In addition to the use of approved cell lines certified cell culture media and transfection reagents approved for vaccine production have to be used. It is anticipated that the resultant 6 + 2 reassortant viruses after those technical modifications will have the same high-growth phenotype as those generated in this study. For the subsequent production of vaccines, embryonated chicken eggs or continuous cell lines can be used. Because of the limited availability of eggs for the production of virus, continuous cell lines are considered an attractive alternative. There is evidence that human viruses propagated in cell lines are more likely than those propagated in eggs to have an HA that resembles the HA of the original human isolate [18–20]. For the production of vaccines, any cell line which allows the efficient replication of influenza virus, such as Vero cells or St. Jude porcine lung (SJPL) cells, can be used [20–22].

We have demonstrated that H3N2 and H1N1 reassortants generated by DNA transfection grow to moderate to high titres in eggs—a growth level equivalent to that observed in classical reassortment techniques. The H6N1 and H9N2 reassortants from subtypes currently circulating in Southeast China grew to high titres in eggs, indicating that our 6 + 2 approach can generate high-yield reassortants of these subtypes. Our findings support the view that the six internal genes of the PR8 virus are important for the high-yield phenotype. Because rgPR8-H5ΔN1 and rgPR8-H6N1 viruses differed only in the *HA* gene, the lower yield of rgPR8-H5ΔN1 virus was caused by the H5Δ hemagglutinin. These results show that not only the internal genes but also the hemagglutinin gene are determinants of the virus yield in eggs. Deletion of basic amino acids (PQ*RERRRKKR* ↓ G → PQ*RETR* ↓ G) of A/Hong Kong/483/97 (H5N1) resulted in a variant that was attenuated in mice [23]. Possibly, the genetic alteration of the H5 molecule reduces the replication efficiency of a virus in mice and in eggs. Further studies using the eight-plasmid system allows to elucidate whether the yield of H5N1 viruses can be increased by genetic manipulation of the H5 (e.g. the addition of amino acids to the connecting peptide) or the *PR8*-genes.

The use of 6 + 2 plasmids to generate 6 + 2 reassortant influenza A viruses eliminates the need for a selection system, thus simplifying the creation of viruses of the desired subtypes. The rapid generation of reassortants and the improved full-length amplification of influenza gene segments by RT-PCR [12] suggest that plasmid collections representing different subtypes of circulating strains, including those isolated from humans and animals, should be created in concert with surveillance studies. We have rescued reassortant PR8 viruses representing 15 HA and nine NA subtypes (data not shown) by using the same technique; therefore, this method is applicable to all influenza virus subtypes. If new pathogenic viruses should emerge, plasmids representing the closest antigenic subtype can be used to produce virus seed suitable for manufacturing a vaccine.

The classical reassortment method currently in use requires lengthy screening and selection procedures that create a 2–3-month lag time between identification of a new strain and the start of vaccine production. If plasmid collections were available, only 1–3 weeks would be needed to generate reassortants by the DNA transfection method. The vaccines could then enter production and become available within about 4–5 months. This 2-month reduction in lag time could be crucial in reducing the spread of a newly emerging pathogenic virus. Further, generation of 6 + 2 viruses by the PR8-plasmid method does not require multiple passages in eggs, as does the classical reassortment method. Viral adaptation to growth in eggs can alter the HA antigens, thereby compromising the protective effect of the vaccine product [24]. Therefore, the *de novo* generation of reassortant influenza viruses by DNA transfection would produce virus seed more quickly, and the product would be more

reproducible and more likely to closely match the circulating strain antigenically.

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References

[1] Subbarao K, Klimov A, Katz J, et al. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 1998;279:393–6.
[2] Claas EC, Osterhaus AD, van Beek R, et al. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 1998;351:472–7.
[3] Peiris M, Yuen KY, Leung CW, et al. Human infection with influenza H9N2. Lancet 1999;354:916–7.
[4] Guan Y, Shortridge KF, Krauss S, Webster RG. Molecular characterization of H9N2 influenza viruses: were they the donors of the internal genes of H5N1 viruses in Hong Kong? Proc Natl Acad Sci USA 1999;96:9363–7.
[5] de Jong JC, Claas EC, Osterhaus AD, Webster RG, Lim WL. A pandemic warning? Nature 1997;389:554.
[6] Kilbourne ED. Future influenza vaccines and the use of genetic recombinants. Bull World Health Organ 1969;41:643–5.
[7] Voeten JT, Brands R, Palache AM, et al. Characterization of high-growth reassortant influenza A viruses generated in MDCK cells cultured in serum-free medium. Vaccine 1999;17(15/16):1942–50.
[8] Li S, Liu C, Klimov A, et al. Recombinant influenza A virus vaccines for the pathogenic human A/Hong Kong/97 (H5N1) viruses. J Infect Dis 1999;179:1132–8.
[9] Neumann G, Watanabe T, Ito H, et al. Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci USA 1999;96:9345–50.
[10] Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, García-Sastre A. Rescue of influenza A virus from recombinant DNA. J Virol 1999;73:9679–82.
[11] Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci USA 2000;97:6108–13.
[12] Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 2001;146:2275–89.
[13] Xu X, Subbarao K, Cox NJ, Guo Y. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology 1999;261:15–9.
[14] Hoffmann E, Stech J, Leneva I, et al. Characterization of the influenza A gene pool in avian species in Southern China: was H6N1 a derivative or a precursor of H5N1? J Virol 2000;74:6309–15.
[15] Webster RG, Guan Y, Peiris M, et al. Characterization of H5N1 influenza viruses that continue to circulate in Geese in Southeastern China. J Virol 2002;76(1):118–26.
[16] Lin YP, Shaw M, Gregory V, et al. Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. Proc Natl Acad Sci USA 2000;97(17):9654–8.
[17] Comai L, Tanese N, Tjian R. The TATA-binding protein and associated factors are integral components of the RNA polymerase-I transcription factor SL1. Cell 1992;68:965–76.
[18] Schild GC, Oxford JS, de Jong JC, Webster RG. Evidence for host-cell selection of influenza virus antigenic variants. Nature 1983;303:706–9.
[19] Robertson JS, Bootman JS, Newman R, et al. Structural changes in the haemagglutinin which accompany egg adaptation of an influenza A(H1N1) virus. Virology 1987;160:31–7.
[20] Seo SH, Goloubeva O, Webby R, Webster RG. Characterization of a porcine lung epithelial cell line suitable for influenza virus studies. J Virol 2001;75(19):9517–25.
[21] Govorkova EA, Murti G, Meignier B, de Taisne C, Webster RG. African green monkey kidney (Vero) cells provide an alternative host cell system for influenza A and B viruses. J Virol 1996;70(8):5519–24.
[22] Kistner O, Barrett PN, Mundt W, et al. Development of a Vero cell-derived influenza whole virus vaccine. Dev Biol Stand 1999;98:101–10.
[23] Hatta M, Gao P, Halfmann P, Kawaoka Y. Molecular basis for high virulence of Hong Kong influenza A viruses. Science 2001;293(5536):1840–2.
[24] Kodihalli S, Justewicz DM, Gubareva LV, Webster RG. Selection of a single amino acid substitution in the hemagglutinin molecule by chicken eggs can render influenza A virus (H3) candidate vaccine ineffective. J Virol 1995;69:4888–97.