Published in final edited form as:

Vaccine. 2010 August 16; 28(36): 5803–5809. doi:10.1016/j.vaccine.2010.06.072.

Repository of Eurasian influenza virus hemagglutinin and neuraminidase reverse genetics vectors and recombinant viruses

J. Keawcharoen¹, M.I.J Spronken¹, O. Vuong¹, T.M. Bestebroer¹, V.J. Munster^{1,§}, A.D.M.E Osterhaus¹, G.F Rimmelzwaan¹, and R.A.M. Fouchier^{1,*}

¹National Influenza Centre and Department of Virology, Erasmus Medical Centre, Rotterdam, The Netherlands

Abstract

Reverse genetics can be used to produce recombinant influenza A viruses containing virtually every desired combination of hemagglutinin (HA) and neuraminidase (NA) genes using the virus backbone of choice. Here, a repository of plasmids and recombinant viruses representing all contemporary Eurasian HA and NA subtypes, H1–H16 and N1–N9, was established. HA and NA genes were selected based on sequence analyses of influenza virus genes available from public databases. Prototype Eurasian HA and NA genes were cloned in bidirectional reverse genetics plasmids. Recombinant viruses based on the virus backbone of A/PR/8/34, and containing a variety of HA and NA genes were produced in 293T cells. Virus stocks were produced in MDCK cells and embryonated chicken eggs. These plasmids and viruses may be useful for numerous purposes, including influenza virus research projects, vaccination studies, and to serve as reference reagents in diagnostic settings.

Keywords

influenza A virus; hemagglutinin; neuraminidase; repository

1. Introduction

Avian influenza A viruses are classified on the basis of antigenic variation of two glycoproteins expressed on the surface of virus particles, hemagglutinin (HA) and neuraminidase (NA). In wild birds throughout the world, influenza A viruses representing 16 HA and 9 NA subtypes have been detected, which can be found in numerous combinations (e.g., H1N1, H16N3) [1,2]. Aquatic birds, in particular those belonging to the orders *Anseriformes* (mostly ducks, geese and swans) and *Charadriiformes* (mostly gulls, terns and shorebirds), form the natural reservoir for low pathogenic avian influenza (LPAI)

© 2010 Elsevier Ltd. All rights reserved

*Corresponding author: R.A.M. Fouchier, PhD. Dept. Virology, Erasmus Medical Centre Rotterdam P.O. Box 2040, 3000 CA Rotterdam Telephone: +31 10 7044066, Fax: +31 10 7044760 r.fouchier@erasmusmc.nl.

§Present address: Laboratory of Virology, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

viruses [1,3]. In these birds, LPAI viruses are generally thought to cause asymptomatic infections. Avian influenza viruses can also infect numerous other species such as domestic birds, humans, pigs, horses, and marine mammals [1]. When LPAI viruses of the H5 and H7 subtypes are introduced into poultry, highly pathogenic avian influenza (HPAI) virus may emerge as the result of the acquisition of a multiple basic amino acid sequence at the HA0 cleavage site that allows systemic virus replication in poultry [4]. During numerous outbreaks in poultry, H7 viruses have been transmitted to humans, in which they frequently caused conjunctivitis and less frequently respiratory illnesses [5]. HPAI H5N1 viruses are still circulating in poultry and wild birds on the eastern hemisphere, predominantly in Asia, Africa, and the Middle East. HPAI H5N1 viruses have been transmitted to humans on numerous occasions, often resulting in severe disease, with a reported case fatality rate of about 60% [6,7]. It is feared that the HPAI H5N1 virus could cause a pandemic if it would acquire the ability to transmit efficiently between humans. Since April 2009, H1N1 viruses of swine origin have been detected in humans, and caused the first pandemic of the twenty-first century [8,9]. This pandemic and the numerous recent influenza virus zoonoses have highlighted the need for reagents and technologies for basic research and for the development of diagnostic tools and vaccines.

In response to the viral zoonoses and presumed pandemic threat(s), numerous large-scale surveillance studies have been initiated in wild and domestic birds around the globe, to screen for the presence of influenza viruses. Virus collections obtained from such studies performed in wild birds in Northern Europe [10–13] were used here to establish a repository of plasmids and recombinant viruses representing all contemporary Eurasian HA and NA subtypes. Prototype strains were selected based on genetic data. The HA and NA genes of the respective strains were cloned in plasmids that can be used to rescue recombinant viruses with reverse genetics techniques or to express HA and NA proteins by transfection [14]. Recombinant viruses, consisting of six gene segments of influenza virus A/Puerto Rico/8/1934 (A/PR/8/34) and the HA and NA gene segments of avian viruses were generated. A/PR/8/34 was chosen because it is attenuated in humans and can therefore be used safely in laboratory settings without strict biosafety regulations [15]. Moreover, A/PR/8/34 is generally the strain of choice to generate vaccine seed strains, because of the relatively high yields of virus production in embryonated chicken eggs or mammalian cell cultures [16,17]. Two sets of recombinant viruses were generated. The first set included all HA and NA subtypes, mostly in combinations as they were found in wild birds. This set may be useful to serve as reference reagents for the determination of influenza A virus subtypes in diagnostic settings [18], to serve as prototype vaccine candidates, as well as for basic research purposes, including structural and functional studies on HA and NA. The second set included recombinant viruses of the H5, H7, and H9 subtypes that are frequently associated with large-scale outbreaks in poultry, and that expressed heterologous NA genes of the N4 and N5 subtypes. N4 and N5 were chosen based on the very low prevalence of these NA subtypes in wild and domestic birds, and very low frequency of detection of these NA subtypes in combination with H5, H7, and H9 [11,19]. As a result, the H5, H7, and H9 viruses with N4 and N5 NA genes may be useful as prototype poultry vaccine strains, to be used following the “Differentiating Infected from Vaccinated Animals (DIVA)” strategy [20].

2. Materials and methods

2.1. Cells

293T cells were cultured in DMEM (Lonza, Breda, The Netherlands) supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/µl streptomycin, 2 mM glutamine, 1 mM sodiumpyruvate and, non-essential amino acids. Madin-Darby Canine Kidney (MDCK) cells were cultured in EMEM (Lonza) supplemented with 10% FCS, 100 IU/ml penicillin, 100

µg/µl streptomycin, 2 mM glutamine, 1,5 mg/ml sodiumbicarbonate, 10 mM Hepes, and non-essential amino acids.

2.2. Viruses

Most of the viruses used in this study were obtained from ongoing influenza virus surveillance programs in wild birds [10,11]. The H14N5 [12] and H15N9 [13] viruses were kindly provided by Dr. R.G. Webster (St. Jude Children's Research Hospital, Memphis, USA). A list of the prototype viruses for each HA and NA subtype, the name of the donor viruses with the corresponding subtype, and the accession numbers for HA and NA genes are provided in Table 1.

2.3. Phylogeny

To generate phylogenetic trees, PhyML was used as a simple maximum likelihood algorithm for analysis of amino acid sequences [21]. All amino acid sequences available from public databases for HA subtypes H1 through H16 and for NA subtypes N1 through N9 of avian origin were downloaded. Only (nearly) full-length sequences were used, and strains with identical sequences were excluded. Furthermore, HA and NA sequences for strains belonging to lineages that were oversampled in the databases, such as those of the Eurasian H5N1 and H9N2 subtypes, were removed. Amino acid sequences were aligned with BioEdit [22], and used as input in PhyML using default settings. Trees were visualized with FigTree [23], and branches with only American lineage HA and NA sequences were collapsed, to only visualize Eurasian avian virus sequences.

2.4. Plasmids

Plasmid pHW2000, a kind gift of Dr. E. Hoffmann (St. Jude Children's Research Hospital, Memphis, USA) was used to generate all reverse genetics vectors [14]. Plasmids containing the gene segments of A/PR/8/34 have been described previously [24]. Each HA and NA gene segment was cloned into the modified version of pHW2000 [24]. To this end, viral RNA was isolated from virus stocks using a High Pure RNA Isolation Kit (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's instructions. The HA and NA genes were amplified by RT-PCR and purified from agarose gels using a mini elute gel extraction kit (Qiagen, Venlo, The Netherlands) according to the instructions of the manufacturer. A second PCR was performed using primers with appropriate restriction sites for cloning into the BsmBI sites of the modified pHW2000 vector. The matrix gene segment from A/Black Headed Gull/Sweden/5/1999 (H16N3) was cloned using the same approach. To construct expression plasmids pCAGGS-PB2, pCAGGS-PB1, pCAGGS-PA and pCAGGS-NP, the open reading frames of the PB2, PB1, PA and NP genes of A/PR/8/34 were cloned into the *Clal-XhoI* or *Nsil-XhoI* restriction sites of pCAGGS. The pCAGGS expression plasmid was kindly provided by Dr. A. Garcia-Sastre (Mount Sinai School of Medicine, New York, USA).

2.5. 293T cell transfections

293T cells were transiently transfected using the calcium phosphate method as described previously [24]. One day before transfection, gelatinized 100 mm dishes were plated with 3×10^6 cells. The next day 50–70% confluent monolayers were transfected with 5 µg of each plasmid encoding the 6 gene segments of the A/PR/8/34 virus and the HA and NA genes representing the subtype of interest (Table 2 and 3). Approximately 16 hours later, cells were washed once with PBS and refreshed with DMEM containing 2% FCS for virus production. Cells were incubated for 72 hours at 37 °C and 5% CO₂, and supernatants were harvested by low-speed centrifugation at 1000 rpm for 10 minutes. Supernatants were used to inoculate embryonated chicken eggs or a confluent monolayer of MDCK cells.

If viruses were not rescued in the first attempt, the second attempt included slight modifications. First, trypsin (Lonza) was added to a final concentration of 175 µg/ml to the virus production medium, and 293T cells were treated with *Vibrio Cholerae* Neuraminidase (VCNA) (Sigma, Zwijndrecht, The Netherlands) before inoculation of MDCK cells or embryonated chicken eggs. VCNA treatment was performed by harvesting both cells and supernatants 72 hours after transfection. The cells were resuspended in 2 ml of DMEM containing 2% FCS and 50 mU of VCNA. After incubation at 37 °C for 45 minutes, the cell suspension was centrifuged for 10 minutes at 1000 rpm and the supernatant was pooled with the previously harvested cell culture supernatant. This virus stock was used to inoculate MDCK cells or embryonated chicken eggs.

To rescue H13N6 and H16N3 viruses, supernatant from the 293T producer cells was discarded and the cells were resuspended in 1 ml of PBS. Embryonated chicken eggs were inoculated with 200 µl of the cell suspension.

2.6. Inoculation of MDCK cells

293T cell supernatants were used to inoculate a confluent monolayer of MDCK cells in T25 cm² flasks. MDCK cells were washed twice with PBS and inoculated with 3 ml of 293T supernatant. After incubation for 1 hour at 37 °C and 5% CO₂, cells were washed with PBS twice and 7 ml of infection medium was added to the cells to allow for virus replication. Infection medium consisted of EMEM (Lonza) supplemented with 100 IU/ml penicillin, 100 µg/µl streptomycin, 2 mM glutamine, 1.5 mg/ml sodiumbicarbonate, 10 mM Hepes, non-essential amino acids, and 17.5 µg/ml trypsin. Three days after inoculation, supernatants were harvested and tested for hemagglutination activity using turkey red blood cells, as an indicator of virus production. 100 µl of each virus supernatant was used to infect another T25 cm² flask of MDCK cells to obtain a second passage.

2.7. Virus titrations

Recombinant viruses were passaged in MDCK cells twice and these supernatants were used to assess infectious virus titres, essentially as described previously [25]. Virus supernatants were 10-fold serially diluted in infection medium. Cells were washed twice with PBS and 100 µl of the diluted virus was used to inoculate confluent monolayers of MDCK cells in 96-wells plates. After incubation for 1 hour at 37 °C and 5% CO₂ cells were washed with PBS twice and 200 µl of infection medium was added to each well. At 3 days post inoculation, the presence of virus was determined by testing for hemagglutination activity with turkey red blood cells and the TCID₅₀ was calculated from 8 replicates according to the method of Spearman-Kärber [26].

2.8. Egg inoculations

Eleven day old embryonated chicken eggs were inoculated with 200 µl of 293T supernatant, 100 µl of MDCK passage two supernatant, or with 200 µl of 293T cell suspension in PBS. After incubation at 37 °C and relative humidity (RH) > 50% in an egg incubator (Neerhof, Lokeren, Belgium) for 48 hours, allantoic fluid was harvested and the presence of virus was determined by testing for hemagglutination activity using turkey red blood cells. All viruses were passaged in eggs twice, unless stated otherwise.

2.9. Egg titrations

Virus titres were obtained from all recombinant viruses after two passages in eggs. Allantoic fluids were ten-fold serially diluted in infection medium and 100 µl of dilutions 10⁻³ to 10⁻⁸ were used to inoculate eggs in duplicate. After incubation at 37 °C and RH > 50% for 48 hours, allantoic fluid was harvested and all dilutions were tested for the presence of virus by

hemagglutination assay using turkey red blood cells. Titres were calculated from 2 replicates, according to the method of Spearman-Kärber [26].

2.10. Confirmation of HA and NA sequences

From all viruses produced, RNA was isolated using a High Pure RNA Isolation Kit. The HA and NA gene segments were amplified by RT-PCR and purified from agarose gels using a mini elute gel extraction kit. This DNA was used for sequence analysis to confirm that the viruses contain the correct HA and NA sequences, using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands) on a 3130xl Genetic Analyzer (Applied Biosystems) according to the instructions of the manufacturer.

3. Results

3.1. Selection of viruses

To establish a repository of reverse genetics vectors containing all HA (H1–H16) and NA (N1–N9) subtypes, a selection was made from viruses isolated during influenza virus surveillance studies in wild aquatic birds in Northern Europe [10,11]. Because viruses of subtypes H14 and H15 have not been detected in recent surveillance studies, older representative strains were used for these virus subtypes [12,13]. To select representative strains, phylogenetic trees were generated that included all unique amino acid sequences for each HA subtype available from public databases, including all sequences for viruses from our own collection. For each HA subtype within this collection, a sequence representative for contemporary Eurasian lineage wild bird LPAI viruses was selected for cloning (Fig. 1 and supplementary figure). Whenever multiple sequences were available, criteria for selecting “representative” sequences were 1) location of the strain sequence within the major group of Eurasian sequences, and 2) location of the strain close to the trunk of the tree. The NA genes were selected to include the full set of 9 NA subtypes, when possible in naturally occurring HA-NA combinations with the selected HA genes. Eight of the 9 NA genes were representative for contemporary Eurasian lineage wild bird LPAI viruses. The N3 gene was chosen from a gull influenza virus, and hence clusters together with other gull N3 sequences in a minor branch of the N3 tree (Fig. 2 and supplementary figure). All 16 HA and 9 NA gene segments were cloned, and plasmid constructs were sequenced to ensure that the nucleotide sequence was identical to the consensus sequence of the original virus isolate. An overview of all HA and NA genes that were cloned with their corresponding donor virus, subtype, and Genbank accession number are shown in table 1.

3.2. Virus production

Two sets of recombinant viruses were generated by reverse genetics techniques. In the first set, the aim was to generate viruses representing all 16 HA subtypes and all 9 NA subtypes. Each HA prototype was combined with a NA gene to generate either natural variants with HA and NA derived from the same virus, or variants with HA and NA derived from two different viruses but in a subtype combination that is also found frequently in nature. Table 2 shows an overview of the reverse genetics vectors and recombinant viruses used for this set. In the second set, the relatively rare NA subtypes N4 and N5 were combined with HA of subtypes H5, H7, and H9 to serve as potential DIVA strains. The selected subtypes, donor viruses and Genbank accession numbers for this set are shown in table 3.

To generate recombinant influenza viruses using reverse genetics, 293T cells were transfected with reverse genetics plasmids containing the desired HA and NA genes and the 6 internal genes of A/PR/8/34. Supernatants were harvested 72 hours after transfection and used to inoculate a confluent monolayer of MDCK cells. Three days after inoculation, supernatants were tested for hemagglutination activity as an indicator of the presence of

virus. In this first attempt, the following viruses were rescued: H2N9, H4N6, H6N1, H8N4, H11N9, and H14N5. Upon a second passage in MDCK cells, recombinant viruses of the H5N2, H5N4, H5N5, H7N4, and H7N5 subtypes were obtained. All other HA-NA combinations did not result in detectable virus production using this method.

In a second attempt to rescue recombinant viruses that were not produced using the simple protocol described above, trypsin was added to the 293T cells and the producer cells were treated with VCNA after three days. Upon a single passage of the 293T cell supernatants in MDCK cells, viruses of the subtypes H3N8, H9N5, H10N2, and H15N9 were rescued. Upon a second passage in MDCK cells, recombinant viruses of the H1N1 and H12N5 subtypes were obtained. All remaining HA-NA combinations still did not result in detectable virus production using this method.

In a third attempt, pCAGGS plasmids expressing PB2, PB1, PA and NP of A/PR/8/34 were co-transfected with the 8 reverse genetics vectors. As in attempt two, trypsin was added to the 293T cells and the producer cells were treated with VCNA after three days. Using this approach, viruses of subtypes H9N2 and H9N4 were rescued upon two passages in MDCK cells. Moreover, after a third passage in MDCK cells, also the virus of the H7N3 subtype was produced. For the remaining 3 virus subtypes, H7N7, H13N6, and H16N3, the 293T cell supernatants were passaged several times in embryonated chicken eggs in an attempt to recover infectious virus. This approach was successful for the H7N7 virus after 4 rounds of propagation in eggs.

Numerous additional attempts to rescue H13N6 and H16N3 viruses using the three methods described above did not result in virus production. In addition, several alternative strategies to rescue these recombinant viruses were tested. First, the 6 internal genes of A/WSN/33 rather than A/PR/8/34 were used to produce recombinant viruses. Second, NA subtypes N6, N8, and N9 were used in combination with H13, since these combinations have also been detected in wild bird surveillance studies. Third, the matrix gene segment of the H16N3 virus was cloned into pHW2000 and co-transfected with plasmids for H16, N3 and the remaining five gene segments of A/PR/8/34, but again no virus was rescued. Finally, 293T cells were transfected as in the first virus rescue attempt, but this time the supernatant was discarded and the cells were resuspended in PBS. Embryonated chicken eggs were inoculated with the cell suspension and this resulted in H13N6 and H16N3 recombinant virus production. Previous experiments showed that H13 and H16 viruses replicated very poorly in MDCK cells (data not shown), and therefore these viruses were not inoculated in MDCK cells.

Thus, using different approaches of increasing complexity, all viruses listed in tables 2 and 3 were produced.

3.3. Virus titrations

All viruses, except H13N6 and H16N3 virus, were propagated in MDCK cells until a second passage was obtained and viral titrations were performed. The viral titres in MDCK cells are shown in table 2 and 3 and range from $10^{3.8}$ to $10^{6.8}$ TCID₅₀/ml.

Embryonated chicken eggs were inoculated with either 293T supernatant or second passage MDCK supernatant. These viruses were propagated in eggs until a second passage was obtained and used for viral titrations in embryonated chicken eggs. Tables 2 and 3 show the viral titres in eggs, ranging from $10^{5.5}$ to $10^{8.5}$ TCID₅₀/ml. Virus titres in embryonated chicken eggs were about 1–2 log higher than those obtained in MDCK cells.

4. Discussion

The classical way to produce influenza virus vaccine seed strains is by reassortment of two viruses in embryonated chicken eggs, one virus with desired antigenic properties of HA and NA, and a second virus with a high yield phenotype, usually A/PR/8/34 [27]. Recent development of reverse genetics techniques facilitated the production of viruses containing the desired HA and NA subtype with any influenza virus backbone [14,28–30]. Recombinant or reassortant viruses can subsequently be propagated on a large scale using embryonated chicken eggs or cell lines such as MDCK cells [31–33].

Vaccine strains can be generated as the need arises in times of outbreaks. However, building a repository of seed strains to choose from ahead of outbreaks is another viable option. Following the latter strategy, the obvious advantage is to have more time to optimize virus yields for each virus subtype prior to outbreaks, and to start vaccine production almost immediately after the start of an outbreak. A downside of using virus repositories could be that the antigenic match between vaccine seed strain and the outbreak would be less than perfect. When outbreaks occur, seed strains from repositories could be used in a first line response, to be followed by a second line response with vaccine strains with a better antigenic match, suited to the needs [34,35]. It should be noted that the seed strains described here were produced in laboratory cell lines. These seed strains would need to be regenerated using validated cell banks before vaccines may be approved for use in humans.

The repository described here was based on viruses of the Eurasian lineage only, and it would be desirable to add strains of the American lineage in the future, as well as other lineages that are not represented in the current dataset, including viruses from other animal species. Repositories like this one should not be regarded as static databases, as old virus lineages may become extinct and new lineages – possibly with new antigenic properties – may arise in the future. Moreover, for economically important virus subtypes such as H5, H7, and H9, it may be appropriate to generate more than one vaccine seed strain when multiple virus lineages are known to co-circulate, as is currently done for viruses of the HPAI H5N1 subtype affecting humans [36]. The latter point is exemplified with the H9 subtype, for which at least three genetic lineages of Eurasian virus sequences can be observed in the tree of Figure 1. For a vaccine seed strain repository, it will further be important to obtain information on the antigenic characteristics of the strains in addition to genetic information. Although the antigenic variation of wild bird viral surface glycoproteins is generally limited, it is possible that particular HA and NA subtypes show sufficient antigenic variation to include more than one strain for that subtype.

Recombinant viruses expressing all HA and NA subtypes were successfully rescued in an A/PR/8/34 virus backbone. For some of the virus subtypes, the addition of trypsin, treatment with VCNA, over-expression of polymerase complex proteins, or repeated passage in MDCK cells or eggs was required for virus rescue. A virus rescue protocol including these additional steps may thus be the most robust protocol in general. However, for the rescue of A/PR/8/34 virus with H13 and H16 HA and N3 NA of gull virus origin, this protocol was still not successful. Numerous additional modifications of the reverse genetics protocol were tested. We used N3, N6, N8, and N9 genes of viruses obtained from mallards as alternatives for the N3 gene obtained from a gull virus, A/WSN/33 and WF10 [37] as alternative virus backbones, and replaced gene segment 7 of A/PR/8/34 with that of a gull virus because HA, NA and MA proteins are known to interact [38]. Finally, embryonated chicken eggs were inoculated with a cell suspension of 293T producer cells, and this resulted in the production of H13N6 and H16N3 recombinant virus. All recombinant viruses, except H13N6 and H16N3, produced in this study could be propagated both in MDCK cells and embryonated chicken eggs, generally at reasonable viral titres. For all recombinant viruses produced here,

virus yields in MDCK were lower than in eggs. Presumably, this was due to the fact that the recombinant viruses contained HA and NA of avian viruses that have avian receptor specificity and are fully adapted to avian host cells. It is possible that repeated passage of the viruses in MDCK cells would increase virus yields in this substrate. Virus yield in eggs of these seed viruses might also be further enhanced by using an A/PR/8/34 strain that is better adapted to eggs as described by Horimoto et al. [39], or by repeated virus passage in eggs. In particular for use as vaccine seed strains, virus titres for some of the strains produced would certainly need improvement.

Beyond the use as vaccine seed strains, repositories like the one described here will be useful for a variety of other purposes. Because the pHW2000 vector designed by Hoffmann and colleagues [14] that was used here is a bidirectional vector containing both an RNA polymerase I promoter to drive synthesis of viral RNA and an RNA polymerase II promoter to drive mRNA synthesis, the plasmids can not only be used to produce recombinant viruses but also to produce HA and NA proteins upon (transient) transfection of eukaryotic cells.

In diagnostic settings, up-to-date reference reagents are needed to validate tests for the detection of specific virus subtypes e.g. by PCR or serology. Moreover, the panels of reagents described here may be useful for fundamental research projects. Since viruses with the A/PR/8/34 virus backbone are generally considered to be attenuated, it may be advantageous to work with recombinant rather than wild type viruses in many diagnostic and research settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Webster for the H14N5 and H15N9 viruses, Dr. Hoffmann for pHW2000, and Dr. Garcia Sastre for the pCAGGS plasmid. We thank C. Baas and P. Lexmond for excellent technical assistance and M. de Graaf for helpful discussions. This study was financially supported by ZonMW grant 91402008, awarded to the Netherlands Influenza Vaccine Research Centre, and National Institute of Allergy and Infectious Diseases–NIH contract HHSN266200700010C.

References

- [1]. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol Rev.* Mar; 1992 56(1):152–79. [PubMed: 1579108]
- [2]. Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, et al. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J Virol.* Mar; 2005 79(5):2814–22. [PubMed: 15709000]
- [3]. Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA. Global patterns of influenza A virus in wild birds. *Science.* Apr 21; 2006 312(5772):384–8. [PubMed: 16627734]
- [4]. Alexander DJ. A review of avian influenza in different bird species. *Vet Microbiol.* May 22; 2000 74(1–2):3–13. [PubMed: 10799774]
- [5]. de Wit E, Kawaoka Y, de Jong MD, Fouchier RA. Pathogenicity of highly pathogenic avian influenza virus in mammals. *Vaccine.* Sep; 2008 12(26 Suppl 4):D54–8. [PubMed: 19230161]
- [6]. de Jong MD, Bach VC, Phan TQ, Vo MH, Tran TT, Nguyen BH, et al. Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. *N Engl J Med.* Feb 17; 2005 352(7):686–91. [PubMed: 15716562]
- [7]. WHO. Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO. 2009

- [8]. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science*. Jul 10; 2009 325(5937):197–201. [PubMed: 19465683]
- [9]. Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD, et al. Pandemic potential of a strain of influenza A (H1N1): early findings. *Science*. Jun 19; 2009 324(5934):1557–61. [PubMed: 19433588]
- [10]. Latorre-Margalef N, Gunnarsson G, Munster VJ, Fouchier RA, Osterhaus AD, Elmerberg J, et al. Effects of influenza A virus infection on migrating mallard ducks. *Proc Biol Sci*. Mar 22; 2009 276(1659):1029–36. [PubMed: 19129127]
- [11]. Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, et al. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. *PLoS pathogens*. May 11.2007 3(5):e61. [PubMed: 17500589]
- [12]. Kawaoka Y, Yamnikova S, Chambers TM, Lvov DK, Webster RG. Molecular characterization of a new hemagglutinin, subtype H14, of influenza A virus. *Virology*. Dec; 1990 179(2):759–67. [PubMed: 2238469]
- [13]. Rohm C, Zhou N, Suss J, Mackenzie J, Webster RG. Characterization of a novel influenza hemagglutinin, H15: criteria for determination of influenza A subtypes. *Virology*. Mar 15; 1996 217(2):508–16. [PubMed: 8610442]
- [14]. 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 U S A*. May 23; 2000 97(11):6108–13. [PubMed: 10801978]
- [15]. Beare AS, Schild GC, Craig JW. Trials in man with live recombinants made from A/PR/8/34 (H0 N1) and wild H3 N2 influenza viruses. *Lancet*. Oct 18; 1975 2(7938):729–32. [PubMed: 52768]
- [16]. Kilbourne ED, Murphy JS. Genetic studies of influenza viruses. I. Viral morphology and growth capacity as exchangeable genetic traits. Rapid in ovo adaptation of early passage Asian strain isolates by combination with PR8. *J Exp Med*. Mar 1.1960 111:387–406. [PubMed: 13755924]
- [17]. Kilbourne ED. Future influenza vaccines and the use of genetic recombinants. *Bull World Health Organ*. 1969; 41(3):643–5. [PubMed: 5309489]
- [18]. WHO. WHO manual on animal influenza diagnosis and surveillance. 2002
- [19]. Krauss S, Walker D, Pryor SP, Niles L, Chenghong L, Hinshaw VS, et al. Influenza A viruses of migrating wild aquatic birds in North America. *Vector Borne Zoonotic Dis*. Fall; 2004 4(3):177–89. [PubMed: 15631061]
- [20]. Capua I, Terregino C, Cattoli G, Mutinelli F, Rodriguez JF. Development of a DIVA (Differentiating Infected from Vaccinated Animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. *Avian Pathol*. Feb; 2003 32(1): 47–55. [PubMed: 12745380]
- [21]. Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol*. Oct; 2003 52(5):696–704. [PubMed: 14530136]
- [22]. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser*. 1999; 41:95–8.
- [23]. Rambaut, A. Tree figure drawing tool. version 1.2.1.. 2009.
- [24]. de Wit E, Spronken MI, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. Efficient generation and growth of influenza virus A/PR/8/34 from eight cDNA fragments. *Virus Res*. Jul; 2004 103(1–2):155–61. [PubMed: 15163504]
- [25]. Rimmelzwaan GF, Baars M, Claas EC, Osterhaus AD. Comparison of RNA hybridization, hemagglutination assay, titration of infectious virus and immunofluorescence as methods for monitoring influenza virus replication in vitro. *J Virol Methods*. Sep; 1998 74(1):57–66. [PubMed: 9763129]
- [26]. Karber. Beitrag zur kollektiven behandlung pharmakologischer reihenversuche *Exp Pathol Pharmacol*. 1931; 162:480–3.
- [27]. Gerdil C. The annual production cycle for influenza vaccine. *Vaccine*. May 1; 2003 21(16):1776–9. [PubMed: 12686093]

- [28]. Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A. Rescue of influenza A virus from recombinant DNA. *J Virol.* Nov; 1999 73(11):9679–82. [PubMed: 10516084]
- [29]. Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, et al. Generation of influenza A viruses entirely from cloned cDNAs. *Proc Natl Acad Sci U S A.* Aug 3; 1999 96(16):9345–50. [PubMed: 10430945]
- [30]. Hoffmann E, Krauss S, Perez D, Webby R, Webster RG. Eight-plasmid system for rapid generation of influenza virus vaccines. *Vaccine.* Aug 19; 2002 20(25–26):3165–70. [PubMed: 12163268]
- [31]. Liu J, Shi X, Schwartz R, Kemble G. Use of MDCK cells for production of live attenuated influenza vaccine. *Vaccine.* Jun 24; 2009
- [32]. Barrett PN, Mundt W, Kistner O, Howard MK. Vero cell platform in vaccine production: moving towards cell culture-based viral vaccines. *Expert Rev Vaccines.* May; 2009 8(5):607–18. [PubMed: 19397417]
- [33]. Halperin SA, Smith B, Mabrouk T, Germain M, Trepanier P, Hassell T, et al. Safety and immunogenicity of a trivalent, inactivated, mammalian cell culture-derived influenza vaccine in healthy adults, seniors, and children. *Vaccine.* Jan 15; 2002 20(7–8):1240–7. [PubMed: 11803087]
- [34]. Jennings LC, Monto AS, Chan PK, Szucs TD, Nicholson KG. Stockpiling prepandemic influenza vaccines: a new cornerstone of pandemic preparedness plans. *Lancet Infect Dis.* Oct; 2008 8(10):650–8. [PubMed: 18922487]
- [35]. de Wit E, Munster VJ, Spronken MI, Bestebroer TM, Baas C, Beyer WE, et al. Protection of mice against lethal infection with highly pathogenic H7N7 influenza A virus by using a recombinant low-pathogenicity vaccine strain. *J Virol.* Oct; 2005 79(19):12401–7. [PubMed: 16160167]
- [36]. WHO. Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use as human vaccines. 2009
- [37]. Perez DR, Lim W, Seiler JP, Yi G, Peiris M, Shortridge KF, et al. Role of quail in the interspecies transmission of H9 influenza A viruses: molecular changes on HA that correspond to adaptation from ducks to chickens. *J Virol.* Mar; 2003 77(5):3148–56. [PubMed: 12584339]
- [38]. Enami M, Enami K. Influenza virus hemagglutinin and neuraminidase glycoproteins stimulate the membrane association of the matrix protein. *J Virol.* Oct; 1996 70(10):6653–7. [PubMed: 8794300]
- [39]. Horimoto T, Murakami S, Muramoto Y, Yamada S, Fujii K, Kiso M, et al. Enhanced growth of seed viruses for H5N1 influenza vaccines. *Virology.* Sep 15; 2007 366(1):23–7. [PubMed: 17651774]

Figure 1.

Analysis of selected HA genes in comparison to HA genes of other Eurasian influenza viruses. For each HA subtype, all available (nearly) full-length amino acid sequences were obtained from public databases, aligned, and analyzed with PhyML as a simple maximum likelihood algorithm. Trees were visualized with FigTree and branches with only American lineage HA sequences were collapsed. For H5N1 and H9N2 subtypes, overrepresented outbreak strains were removed before building the tree. HA sequences selected for cloning and recombinant virus production are indicated with arrowheads. Strain names are only shown in the accompanying supplementary figure.

Figure 2.

Analysis of selected NA genes in comparison to NA genes of other Eurasian influenza viruses. For each NA subtype, all available (nearly) full-length amino acid sequences were obtained from public databases, aligned, and analyzed with PhyML as a simple maximum likelihood algorithm. Trees were visualized with FigTree and branches with only American lineage NA sequences were collapsed. For H5N1 and H9N2 subtypes, overrepresented outbreak strains were removed before building the tree. NA sequences selected for cloning are indicated with arrowheads. Strain names are only shown in the accompanying supplementary figure.

Table 1

HA and NA subtypes used in this study.

HA/NA subtype	Virus name	Virus subtype	Accession number
H1	A/Common Teal/Netherlands/10/00	[H1N1]	CY060178
H2	A/Mallard/Netherlands/5/99	[H2N9]	CY064950
H3	A/Mallard/Sweden/50/02	[H3N8]	CY060308
H4	A/Mallard/Netherlands/1/99	[H4N6]	CY060241
H5	A/Mallard/Netherlands/3/99	[H5N2]	AY684894
H6	A/Mallard/Sweden/81/02	[H6N1]	CY060383
H7	A/Mallard/Netherlands/12/00	[H7N3]	AY338460.1
H8	A/Mallard/Sweden/24/02	[H8N4]	CY060249
H9	A/Eurasian wigeon/NL/4/05	[H9N2]	HM136574
H10	A/Mallard/Sweden/51/02	[H10N2]	HM136575
H11	A/Shoveler/Netherlands/18/99	[H11N9]	EF200063
H12	A/Mallard/Sweden/86/03	[H12N5]	CY060390
H13	A/Black Headed Gull/Sweden/1/99	[H13N6]	AY684887.1
H14	A/Mallard/Gurjev/263/82	[H14N5]	M35997.1
H15	A/Shearwater/West Australia/2576/79	[H15N9]	L43917.1
H16	A/Black Headed Gull/Sweden/5/99	[H16N3]	AY684891.1
N1a	A/Common Teal/Netherlands/10/00	[H1N1]	CY060180
N1b	A/Mallard/Sweden/81/02	[H6N1]	CY060384
N2	A/Mallard/Netherlands/3/99	[H5N2]	CY064944
N3	A/Black Headed Gull/Sweden/2/99	[H16N3]	AY684902.1
N4	A/Mallard/Sweden/24/02	[H8N4]	CY064796
N5	A/Mallard/Sweden/86/03	[H12N5]	CY060392
N6	A/Mallard/Netherlands/1/99	[H4N6]	CY060243
N7	A/Chicken/Netherlands/1/03	[H7N7]	AY340077.1
N8	A/Mallard/Sweden/50/02	[H3N8]	CY060310
N9	A/Mallard/Sweden/36/03	[H11N9]	HM136576

Table 2

Virus subtypes rescued in an A/PR/8/34 backbone and the viral titres obtained in MDCK cells and embryonated chicken eggs.

Subtype	HA donor	NA donor	Titre MDCK	Titre Egg
H1N1	A/Common Teal/Netherlands/10/00	A/Common Teal/Netherlands/10/00	10 ^{6.3}	10 ^{8.0}
H2N9	A/Mallard/Netherlands/5/99	A/Mallard/Sweden/36/03	10 ^{6.3}	10 ^{8.0}
H3N8	A/Mallard/Sweden/50/02	A/Mallard/Sweden/50/02	10 ^{6.5}	10 ^{7.0}
H4N6	A/Mallard/Netherlands/1/99	A/Mallard/Netherlands/1/99	10 ^{6.8}	10 ^{7.5}
H5N2	A/Mallard/Netherlands/3/99	A/Mallard/Netherlands/3/99	10 ^{6.5}	10 ^{8.5}
H6N1	A/Mallard/Sweden/81/02	A/Mallard/Sweden/81/02	10 ^{5.9}	10 ^{8.0}
H7N3 *	A/Mallard/Netherlands/12/00	A/Black Headed Gull/Sweden/2/99	10 ^{4.0}	10 ^{5.5}
H7N7 **		A/Chicken/Netherlands/1/03	10 ^{6.0}	10 ^{7.5}
H8N4	A/Mallard/Sweden/24/02	A/Mallard/Sweden/24/02	10 ^{3.8}	10 ^{6.5}
H9N2	A/Eurasian wigeon/Netherlands/4/05	A/Mallard/Netherlands/3/99	10 ^{6.5}	10 ^{7.5}
H10N2	A/Mallard/Sweden/51/02	A/Mallard/Netherlands/3/99	10 ^{5.3}	10 ^{8.0}
H11N9	A/Shoveler/Netherlands/18/99	A/Mallard/Sweden/36/03	10 ^{5.6}	10 ^{7.0}
H12N5	A/Mallard/Sweden/86/03	A/Mallard/Sweden/86/03	10 ^{4.4}	10 ^{7.0}
H13N6	A/Black Headed Gull/Sweden/1/99	A/Black Headed Gull/Sweden/2/99	ND	10 ^{8.5}
H14N5	A/Mallard/Gurjev/263/82	A/Mallard/Sweden/86/03	10 ^{6.5}	10 ^{6.5}
H15N9	A/Shearwater/West Australia/2576/79	A/Mallard/Sweden/36/03	10 ^{5.4}	10 ^{8.0}
H16N3	A/Black Headed Gull/Sweden/5/99	A/Black Headed Gull/Sweden/2/99	ND	10 ^{6.5}

ND Not done

* The H7N3 was positive after 3 passages in MDCK cells.

** The H7N7 was positive after 4 passages in eggs.

Table 3

DIVA strains and the titres obtained in MDCK cells and eggs.

Subtype	HA donor	NA donor	Titre MDCK	Titre Egg
H5N4	A/Mallard/Netherlands/3/99	A/Mallard/Sweden/24/02	10 ^{6.0}	10 ^{7.5}
H5N5		A/Mallard/Sweden/86/03	10 ^{5.9}	10 ^{8.5}
H7N4	A/Mallard/Netherlands/12/00	A/Mallard/Sweden/24/02	10 ^{4.0}	10 ^{7.5}
H7N5		A/Mallard/Sweden/86/03	10 ^{4.4}	10 ^{7.0}
H9N4	A/Eurasian wigeon/Netherlands/4/05	A/Mallard/Sweden/24/02	10 ^{4.3}	10 ^{6.5}
H9N5		A/Mallard/Sweden/86/03	10 ^{5.1}	10 ^{7.5}