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Improvement of H5N1 influenza vaccine viruses: Influence of internal gene segments of avian and human origin on production and hemagglutinin content

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Abstract

The H5N1-clade 1 influenza vaccine strain NIBRG-14 produces exceptionally low amounts of antigen, a problem recently encountered also for initial pandemic H1N1-2009 vaccine seeds. Here, we report on a strategy that may contribute to overcome this obstacle. Influenza vaccine viruses usually consist of two segments coding for the antigenic HA and NA proteins of a wild-type strain and the six residual internal gene segments of the vaccine donor strain A/PR/8/34 (PR8). To enhance the antigen yield from H5N1 vaccine virus we generated by reverse genetics a set of PR8-based reassortant viruses expressing the HA and NA segments of the prototypic strain A/Vietnam/1203/2004 and additional replacements of the internal M or PB1 genes of PR8. The reassortants were compared to the parental PR8 and H5N1 viruses in terms of growth in embryonated chicken eggs and the amount of incorporated antigenic HA protein. Compared to NIBRG-14, three out of six viruses displayed an increased replication in embryonated chicken eggs and higher HA content that was also maintained after ether/detergent extraction of virions. Electron microscopic analysis showed that the reassortment hardly affected particle shape and size. Two selected H5N1 reassortant viruses were investigated concerning their pathogenicity in ferrets and found to behave as low pathogenic as the PR8 donor strain. In conclusion, this study shows that replication and antigen content of PR8-derived H5N1 influenza vaccine viruses can be improved by incorporation of heterologous internal gene segments without compromising their attenuated character.

1. Introduction

After the first infections of humans by influenza A viruses of the H5N1 subtype in Hong Kong in 1997 considerable efforts have been made to develop an effective vaccine against H5N1 influenza in humans. As of August 2010, a total of 504 human H5N1 cases have been registered by WHO with a stunning case fatality rate of about 60% (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_08_12/en/index.html). So far, the low transmissibility among humans prevented epidemic or pandemic spread of H5N1 viruses [1] and [2]. However, a novel swine-origin H1N1 influenza virus (S-OIV, here referred to as H1N1-2009) appeared in North America in early 2009 and caused infections on a global scale within a few months triggering hasty developments of corresponding pandemic vaccines [3] and [4]. In June 2009 the WHO raised the pandemic alert to phase 6. Infections by the H1N1-2009 virus had been detected in at least 214 countries and territories (http://www.who.int/csr/don/2010_08_06/en/index.html) before the post-pandemic status was declared on August 10th 2010.

Inactivated influenza A vaccines are usually generated with the apathogenic PR8 strain, known to grow to high titres in embryonated chicken eggs, by using either classical reassortment or reverse genetic methods [5], [6] and [7]. For classical reassortment, embryonated chicken eggs are co-infected with a circulating wild-type (WT) virus and the PR8 vaccine donor strain. Progeny viruses are screened for 6 + 2 reassortants containing the six internal gene segments of PR8 and the antigenic

HA and NA genes coding for the surface glycoproteins of the WT strain [8]. The use of reverse genetics has the advantage that reassortant viruses with the desired gene constellation can be rescued directly from tissue culture cells transfected with cloned viral cDNAs [9], [10] and [11]. In addition, this procedure allows the removal of virulence determinants from viral proteins such as an oligobasic cleavage site within the HA protein. Many commercial manufacturers produce well tolerated seasonal “split vaccines” in which the bulk of vaccine virus is chemically inactivated and extracted with detergents and organic solvent to separate the immunogenic surface glycoproteins from reactogenic compounds [12] and [13]. The currently available H5N1 vaccines were shown to be safe and immunogenic when formulated as split-, adjuvanted whole virion- or subvirion vaccine [14], [15] and [16].

The influenza A/Vietnam/1203/2004 (VN1203) and A/Vietnam/1194/2004 (VN1194) viruses were recommended by WHO as prototypic clade 1 H5N1 strains for the generation of pre-pandemic vaccines in the year 2005. One of the first prototypic H5 vaccine strains termed NIBRG-14 was based on the PR8 backbone and carried the two gene segments encoding for HA and NA of VN1194, in which the HA gene had been modified to encode a monobasic cleavage site [17]. However, when NIBRG-14 was used for vaccine preparations in embryonated chicken eggs antigen yields were reported to be drastically lower compared to yields achieved with seasonal vaccine strains [18]. This was possibly caused by suboptimal interaction(s) between internal PR8 gene products and the heterologous H5N1 surface glycoproteins. A subsequent study confirmed the low HA content of NIBRG-14 compared to two other H5 vaccine and wild type viruses [19]. A similar problem was recently also encountered for reassortant vaccine seeds targeting the pandemic H1N1-2009 virus [20]. The poor growth of the initial pandemic H1N1-2009 vaccine viruses resulted in a considerable shortage and delay in the distribution of H1N1 vaccine extending the period of time during which risk groups could not be protected by immunoprophylaxis. For an optimal control of pandemic and seasonal influenza it is therefore needed to explore strategies to overcome such a potential shortage in vaccine production.

One possibility to enhance the antigen yield of influenza vaccine strains could be the substitution of internal genes of the PR8 donor such as the polymerase complex or M segments. The M1 protein is located underneath the particle membrane [21] and is described to interact with the virus surface glycoproteins as well as with the vRNPs in the virus particle [22] and [23]. This interaction is thought to be essential for assembly and budding of virus particles [22]. Moreover, the cytoplasmic tail of the M2 protein was found to be involved in the production of new virus particles and the efficient packaging of virus genomes [24] and [25]. As the PR8-M proteins may not interact optimally with the HA and NA proteins of H5N1 strains we hypothesized that M segments from other strains could influence the growth and possibly antigen yield of H5N1 influenza vaccine viruses. Moreover, several high-yielding H3N2 vaccine viruses generated by classical reassortment had retained the PB1 segment of the seasonal virus indicating this incorporation to be beneficial for virus production [26]. Also, the H1N1-2009 seed strain A/reassortant/NYMC X-179A harboured the PB1 gene segment and the surface glycoprotein genes of the prototypic A/California/07/2009 strain (Genbank accession GQ214335).

With those findings in mind we generated by reverse genetics a set of PR8-based reassortant viruses carrying the HA and NA gene segments of VN1203, in which also the internal M or PB1 segments were replaced (5 + 2 + 1 reassortants). This set of viruses was compared to the NIBRG-14 vaccine strain in terms of replication in embryonated chicken eggs and HA content. Overall, we identified two H5N1 candidate vaccine viruses with enhanced growth and incorporated HA protein, which was also maintained under conditions of split vaccine preparation. Infections of ferrets with these two selected viruses demonstrated that they behaved as benign as the parental A/PR/8/34 virus confirming their suitability for vaccine production under normal biosafety level. Hence, this study shows that modifications of the PR8 background can optimize influenza vaccine seed viruses.

2. Materials and methods

2.1. Cells and viruses

293T cells were grown in Dulbecco's modified eagle medium (D-MEM) supplemented with 10% fetal calf serum, l-glutamine (2 mM) and penicillin/streptomycin (100 µg/ml). Madin Darby canine kidney cells (MDCK II) were grown in minimum essential medium (MEM) supplemented as described above. All the cells were maintained at 37 °C and 5% CO_2. The H5N1-clade 1 vaccine strain NIBRG-14 and the A/Vietnam/1203/2004 WT virus were obtained from the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK). Recombinant A/Vietnam/1203/04 wild type viruses with multi- (VN1203-hp) or monobasic (VN1203-lp) cleavage site were generated by reverse genetic methods in our laboratory [27]. Site-directed mutagenesis was used to replace the multibasic cleavage within in the corresponding HA plasmid from “RERRRKKRG” to the monobasic sequence “RETRG”.

2.2. Plasmids

RNA polymerase I (pol I)-driven expression plasmids for the eight gene segments of influenza A/PR/8/34 virus, pPol I plasmids encoding the vRNAs of the VN/1203 NA and HA with a monobasic cleavage site (HA-VN1203-lp) and pCAGGS plasmids expressing the A/PR/8/34 viral proteins PB2, PB1, PA and NP proteins were kindly provided by Peter Palese (Mt. Sinai School of Medicine, New York) [11]. The vector containing the M gene of A/chicken/R28/Germany/2003 (H7N7) was obtained from the Institute of Virology, University of Marburg, Germany. The cDNA of the A/Panama/2007/99M gene (Genbank accession DQ487338) was cloned into the pHW2000 expression vector [9] by standard methods.

2.3. Production of recombinant viruses

Reassortant and recombinant viruses were generated by the 12-plasmid reverse genetic system as described by Fodor et al. [11]. Briefly, plasmids were transfected into 293T cells with Lipofectamin 2000 (Invitrogen) according to manufacturer's instructions and cells were incubated for two days at 37 °C. Subsequently, supernatants of transfected 293T cells were harvested and inoculated in 10-day-old embryonated chicken eggs. Eggs were incubated for 48 h at 37 °C and the allantoic fluid was harvested, clarified and tested for hemagglutination and plaque assays.

2.4. Quantification of virus titres

To determine the growth capability of a given virus five specific pathogen-free 11-day-old embryonated chicken eggs (Lohmann Tierzucht, Cuxhaven, Germany) were inoculated with 10e3 PFU from the corresponding second passage after virus rescue. Eggs were incubated for 48 h at 37 °C. For the high and low pathogenic versions of the VN1203 virus incubation time was decreased to 41 h to avoid excessive cytopathic effects. The allantoic fluids of the five inoculated eggs were harvested and clarified by centrifugation (3000 rpm, 10 min, 4 °C) and serial dilutions of each virus stock were inoculated into 11-day-old embryonated chicken eggs to determine the minimal egg infectious dose (EID_{50}/ml). After a 48 h incubation period (41 h for recombinant VN1203 viruses), the allantoic fluids were collected and assayed for the presence of virus by hemagglutination. The EID_{50}/ml was determined by the formula of Reed and Muench [28]. To determine virus titres in organ homogenates, MDCK II cells were seeded in 96-well plates (flat bottom) one day prior to infection. A series of log step dilutions of homogenized organ samples or swabs were made in infection medium (MEM, 0.2% bovine albumin, 20 mM glutamine, 1 µg/ml TPCK-treated trypsin plus antibiotics). Cells were washed twice and infected with the homogenates using a total of 5 replicates per dilution. Tissue cultures were incubated at 37 °C for 72 h, fixed with paraformaldehyde and stained with crystal violet. The tissue culture infectious dose ($TCID_{50}$) values were calculated using the method of Reed and Muench [28].

2.5. Deglycosylation and analysis of virion proteins

Viruses were purified from the allantoic fluids of 11-day-old embryonated chicken eggs by low-speed clarification (3000 rpm, 10 min, 4 °C) followed by centrifugation through a 25% sucrose cushion in a SW28 rotor (24,000 rpm, 90 min, 4 °C). The virus pellet was resuspended in PBS and the total amount of purified virion proteins was determined by standard BCA protein assay (Thermo Scientific). 20 µg of total protein was denatured according to the manufacturer's instructions and subsequently digested with 10 U PNGase F (New England Biolabs) for 16 h at 37 °C as described [19]. Samples were mixed after overnight digestion with loading dye and heated to 95 °C for 5 min prior to separation by SDS gel electrophoresis and visualization by Coomassie staining was done by using The Li-Cor Odyssey imaging system and the application software version 2.1 (Li-Core Biosciences) were used for quantification of single protein bands. Protein amounts were adjusted to equal amounts of NP protein and HA/NP ratios were calculated.

2.6. Ether/detergent extraction of viral glycoproteins

Virions were purified from allantoic fluid as described above and resuspended in PBS followed by addition of an equal volume of an ether/Tween-80 (0.01%) mixture [29]. The samples were stirred for 30 min at room temperature and were subsequently centrifuged for 2 min at 12,000 rpm. The aqueous phase was collected, treated with PNGase F as described above and the viral proteins were separated and visualized by SDS-PAGE and Coomassie staining.

2.7. Analysis of virion particles by immunogold electron microscopy

Virus particles were concentrated by ultracentrifugation as described above and fixed with a final concentration of 2% paraformaldehyde in 0.05 M HEPES buffer. Virus particles were adsorbed onto a carbon reinforced plastic film that was supported by a copper grid, by putting the grid on a drop of the virus suspension for 10 min. After adsorption of virus particles, grids were transferred over a sequence of droplets (30 µl) of the following solutions for immuno-negative staining: 50 mM glycine (in PBS), 0.1% bovine serum albumin (BSA) diluted in PBS, primary antibody (diluted in 0.1% BSA), 0.1% BSA (3 times), secondary antibody (coupled to 5 nm of gold and diluted with 0.1% BSA), PBS (3 times), double distilled water (5 times), and uranyl acetate (1% in water). Incubation time at room temperature was 10 min for glycine, BSA and secondary antibody and 15 min for the primary antibody. All washing steps were performed for 1 min. The primary antibody was a purified monoclonal mouse antibody raised against H5-HA (anti-H5HA-22/03/D9-3/D10) that was kindly provided by the National Reference Centre for Influenza at the Robert Koch-Institute. Grids were inspected with a transmission electron microscope (Tecnai12 BioTwin; FEI) and images of virus particles were recorded with a 1k (MegaviewIII; OSIS) or a 2k CCD-camera (TVIPS F214; Tietz). Area and circumference of randomly selected virus particles were measured as projected by the microscope using the iTEM-software (OSIS, Germany) and manual segmentation of virus particles.

2.8. Infection of ferrets

Female outbred ferrets were obtained from Schimmel (the Netherlands). The ferret safety testing was conducted according to WHO guidelines [30] and was approved by a local committee and done in accordance with national guidelines for the care and use of laboratory animals. All ferrets were kept in quarantine 14 days prior to infection and were screened for Aleutian Disease and previous influenza virus infections. For the study 4 groups of 6 ferrets between 16 and 32 weeks of age were intranasally infected under anaesthesia with 0.5 ml containing 10^7 $TCID_{50}$ of either the high pathogenic A/Vietnam/1203/2004 H5N1 WT strain, the reassortant PR8-HA VN1203 (R#2), the reassortant PR8-HA/NA VN1203-M Pan99 (R#4) or the A/PR/8/34 vaccine donor strain. Each group was divided into two subgroups (A and B) and nasal swabs were obtained from all animals prior to infection and from day one until day 3 post infection (p.i.) for virological analysis. Animals of subgroup A were sacrificed at day 3 p.i. and samples were taken from the lungs, brain, spleen and nasal turbinates for storage at −70 °C and subsequent virological analysis. Animals of subgroup B were followed for disease

development for two weeks. To this end, animals were weighed and clinical signs (respiratory disease, activity and neurological symptoms) were recorded daily from day −2 until day 3 p.i., thereafter every second day. Body temperature was monitored every 30 min by a transplanted temperature logger (DST micro-T, StarOddi). For virus titration, frozen samples (day 3 p.i.) were thawed, weighed and transferred to lysing D matrix tubes (MP Biomedical) containing 1.4 mm ceramic spheres (lung, brain, spleen) or lysing A matrix tubes containing garnet shards and a 1/4″ ceramic sphere (trachea, nasal turbinate). 0.2 ml PBS/0.1 g tissue was added to the tubes and organs were homogenized in a FastPrep24 homogenizer (MP Biomedical). Thereafter, tubes were centrifuged twice and clarified supernatants were used immediately for virus titration as described above.

3. Results

3.1. Generation of recombinant influenza A viruses

Using plasmid-based reverse genetics [11] we generated a panel of PR8-based reassortant viruses with glycoprotein genes of the prototypic H5N1 strain VN1203 and additional replacement by the M or PB1 segments derived from human or avian strains (Fig. 1). This set included a recombinant PR8 WT virus (PR8; #1), the reassortant #2 encoding the HA protein of VN1203 with an engineered monobasic cleavage site and seven segments of PR8 (PR8-HA VN1203), and the reassortant #3 consisting of six internal PR8 segments and both viral surface glycoproteins of VN1203 (PR8-HA/NA VN1203). The reassortants #4–#6 carry replacements of the M segment derived from human or avian strains, respectively, in addition to the VN1203 surface glycoproteins genes: Reassortant #4 contains the M segment of the seasonal A/Panama/2007/99 (Pan99) virus (PR8-HA/NA VN1203-M Pan99), reassortant #5 carries the M segment of A/chicken/Germany/R28/2003 (H7N7) (PR8-HA/NA VN1203-M R28), whereas reassortant #6 expresses the M segment of VN1203 (PR8-HA/NA/M VN1203). Reassortant #7 comprises the PB1, HA and NA gene segments of VN1203 (PR8-HA/NA/PB1 VN1203). The prototypic NIBRG-14 H5N1 vaccine virus encoding the glycoprotein genes of VN1194 was obtained from NIBSC and was included in our analysis as reassortant #8. Reassortant #9 includes the H3N2 glycoprotein genes of the seasonal Pan99 strain in the PR8 background (PR8-HA/NA Pan99) and served as a control. Furthermore, two recombinant H5N1 viruses with all eight segments of the VN1203 virus were generated, which differed only in the monobasic (VN1203-lp; #10) or multibasic cleavage site in the HA (VN1203-hp; #11). All viruses were passaged twice in embryonated chicken eggs before their growth behaviour and protein content were examined.

3.2. Identification of H5N1 reassortant viruses with enhanced growth in embryonated chicken eggs compared to NIBRG-14

High titre growth in embryonated chicken eggs is a prerequisite to achieve high antigen yields from influenza vaccine viruses. The growth capabilities of the generated recombinant/reassortant viruses in chicken eggs were compared with the parental recombinant PR8, the NIBRG-14 vaccine strain and the high- and low-pathogenic VN1203 viruses. Fig. 2 shows the mean viral titres for each virus determined in five independent experiments. The six reassortant viruses PR8-HA VN1203 [#2] (2.8×10^9 EID_{50}/ml), PR8-HA/NA VN1203 [#3] (3.1×10^9 EID_{50}/ml), PR8-HA/NA VN1203-M Pan [#4] (2.7×10^9 EID_{50}/ml), PR8-HA/NA/M VN1203 [#6] (1.4×10^9 EID_{50}/ml) as well as the recombinant PR8 [#1] (3.6×10^9 EID_{50}/ml) and A/Vietnam/1203/2004-lp viruses [#10] (2.1×10^9 EID_{50}/ml) replicated to higher mean titres than NIBRG-14 [#8] (1.2×10^9 EID_{50}/ml). In contrast, the reassortant viruses with the M segment of A/chicken/Germany/R28/2003 [#5], the PB1 gene of VN1203 [#7], or the glycoprotein genes of A/Panama/2007/1999 [#9] replicated to lower mean titres (#5: 7.3×10^8 EID_{50}/ml; #7: 4.2×10^8; #9: 6.6×10^8 EID_{50}/ml). The recombinant A/Vietnam/1203/2004-hp [#11] showed the lowest growth among all tested viruses (2.5×10^8 EID_{50}/ml). These results suggest that the growth of PR8 derived 6 + 2 reassortant viruses can be modulated by replacement of an internal gene with a homologous segment from a different virus strain.

3.3. Comparison of surface glycoprotein incorporation in reassortant virions

For high vaccine yield it is required that the corresponding virus incorporates a high amount of immunogenic HA and NA surface glycoproteins, in addition to efficient viral growth. We therefore evaluated whether the exchange of internal gene segments would influence this property. To compare the levels of virion proteins within the set of recombinants we purified viruses from egg allantoic fluid by centrifugation through a sucrose cushion followed by SDS gel analysis. Fig. 3A shows that HA and NA proteins are highly glycosylated and therefore not easily distinguishable by SDS-gel analysis without modification. Hence, the oligosaccharide side chains were removed from HA and NA in the purified virions by deglycosylation with PNGaseF prior to analysis by gel electrophoresis and total protein staining. The amounts of the virion preparations were adjusted to equal NP protein signals before they were separated by standard gel electrophoresis (Fig. 3B). This allowed the quantification of single viral protein bands by densitometric analysis and enabled the calculation of HA1/NP ratios that was arbitrarily set to 1 for NIBRG-14. Subsequently, the *x*-fold differences between NIBRG-14 and other investigated viruses were determined.

Here it was found that the two reassortants #4 (PR8-HA/NA VN1203-M Pan99) and #5 (PR8-HA/NA VN1203-M R28), containing a replacement of the M segment exhibited 3.2- and 3.7-fold increases in the HA1/NP ratio compared to NIBRG-14, which were statistically significant (Fig. 3C). Slightly lower enhancements in the HA1/NP ratio were found for reassortants #6 (PR8-HA/NA/M VN1203), #7 (PR8-HA/NA/PB1 VN1203), #2 (PR8-HA VN1203) and the parental PR8 virus (2.3-, 1.9-, 1.7-, and 1.8-fold, respectively). Additionally, we included the recombinant VN1203-lp virus in our experiments to investigate the HA1/NP ratio in a mere H5N1 virus in comparison to the reassortant viruses. We found that the recombinant wild type virus differed only slightly in its HA1/NP ratio (1.7-fold) from NIBRG-14. Together these data clearly indicate that the amount of virion-incorporated H5-HA protein is influenced by the replacements of the M and PB1 segments with homologs of other strains.

3.4. Effect of ether/detergent extraction on HA content of reassortant viruses

Many influenza vaccine preparations involve an extraction step with ether/detergent solution in which reactogenic compounds are removed from the antigenic glycoproteins. However, this procedure might also influence the relative amount of HA in the soluble fraction. We therefore addressed the question whether the enhanced HA/NP ratio observed for some of the reassortant viruses was retained in the soluble antigen preparation after such a treatment. Purified virus suspensions of the selected viruses #1, #2, #4, #8 and #10 were extracted with an ether/Tween-80 (0.01%) solution and oligosaccharide chains were removed from the HA and NA proteins in the soluble phase by PNGase digestion. The SDS gel analysis of the samples showed that the favourable HA/NP ratio particularly of reassortant #4 was maintained during the extraction procedure (Fig. 3D).

3.5. Replacements of M segments do not influence particle shape of H5N1 reassortant influenza viruses

It has been described that the viral morphogenic M1 and M2 proteins influence the structure of influenza A virus particles by specifying a spherical or rather filamentous virion shape [31], [32] and [33]. We therefore assessed by immuno-negative staining electron microscopic analyses whether alterations in the HA content of the generated reassortant viruses were associated with changes in the principle particle morphology or size (area and circumference). This analysis was conducted with purified virions from NIBRG-14, the reassortants #2 (PR8-HA VN1203), #4 (PR8-HA/NA VN1203-M Pan99) and #5 (PR8-HA/NA VN1203-M R28), as well as from the parental PR8 (#1) and VN1203-lp viruses (#10). Immunolabelling of virus particles with primary anti-H5-HA antibody and a secondary immunogold labelled antibody was done to visualize the presence of HA at the virus surface. Fig. 4 shows representative micrographs for the recombinant PR8 (#1), the VN1203-lp (#10), NIBRG-14 (#8) and the PR8-HA/NA VN1203-M Pan (#4) viruses. As expected, the H5-HA antibody stained all H5 subtype viruses with the exception of PR8 that belongs to the H1 subtype. In general, there were no major differences in the spherical shapes of the viruses. Analysis of one hundred particles for each virus showed that the H5N1 reassortants #2, #4 and #5 exhibited a slightly higher mean virion area (8124–8774 nm^2) compared to PR8 (7244 nm^2) and NIBRG-14 (7809 nm^2) with little

differences in the mean circumferences (Table 1). Virions of the VN1203-lp virus (#10) displayed the highest mean area value (8979 nm^2). Overall, these experiments showed that the reassorted gene segments did not cause major differences in virion shape or size.

3.6. Integrative assessment of H5N1 reassortant viruses

The set of reassortants contained representatives with both higher and lower EID_{50}/ml titres and HA/NP ratios, respectively, than NIBRG-14. These two parameters were arbitrarily set to 1 for NIBRG-14. To identify the most promising candidates for high yielding strains we first calculated the relative EID_{50}/ml value and the relative HA1/NP ratio of each virus in comparison to NIBRG-14. Subsequently, these two parameters were multiplied to obtain a total relative score for each virus. These estimates are depicted in Table 2 showing that the replacement of the PR8-M segment with the M segment of Pan99 in reassortant #4, which resulted in an increased HA1/NP ratio and decent viral growth, achieved the highest score of 7.4. The two other viruses with heterologous M segments, reassortants #5 (PR8-HA/NA VN1203-M R28) and #6 (PR8-HA/NA/M VN1203), respectively, also showed increased HA1/NP ratios. The high HA content was, however, accompanied by less pronounced mean EID_{50}/ml values that still resulted in improvement factors of 2.2-fold and 2.8-fold towards NIBRG-14. The 7 + 1 reassortant containing the HA segment of VN1203 in the PR8 background (#2) replicated to similar high titres, but displayed a slightly lower HA1/NP ratio leading to an improvement score of 4.1. Overall, this analysis identified the reassortants #2 and #4 as the most promising candidates with enhanced growth and HA content for further development.

3.7. Selected H5N1 reassortant viruses are strongly attenuated in ferrets

PR8-based reassortant viruses to be used for the regular production of inactivated vaccine towards high pathogenic H5N1 strains need to be shown to behave non pathogenic [30]. The reduced pathogenicity of the reassortants #2 and #4 was first indicated by their inability to form plaques in tissue culture in the absence of trypsin (data not shown). To assess the virulence potential of the two candidate viruses #2 and #4 *in vivo* we compared their pathogenicity in ferrets to the highly pathogenic H5N1 wild-type strain VN1203 and the parental PR8. Four groups of 6 ferrets were infected intranasally with 0.5 ml containing 10^7 $TCID_{50}$ of either A/Vietnam/1203/2004 (positive control), A/PR/8/34 (negative control), the 7 + 1 reassortant (PR8-HA VN1203, #2) or the 5 + 2 + 1 reassortant (PR8-HA/NA VN1203-M Pan, #4). Virus titres in nasal swabs were determined from day 1 to 3. Each group was subdivided into subgroups A and B, in which animals from subgroup A were sacrificed on day 3 to determine viral loads in organ samples, whereas ferrets in subgroup B were followed for clinical disease for 14 days. In all analysed nasal swabs taken during the first three days, both parental viruses and the two reassortants were detected (Fig. 5). Intranasal virus titres in animals infected with the two reassortants or PR8 peaked on day 2 but were strongly reduced on day 3 p.i. In contrast, intranasal VN1203 titres remained at high level throughout day 3. As expected, the VN1203 WT virus was re-isolated from the brains of three animals (Fig. 6A) and the lungs of two ferrets (Fig. 6B) reflecting the pathogenic phenotype. Contrary, neither the parental PR8 vaccine donor nor the two reassortant viruses PR8-HA/NA VN1203-M Pan (R#4) and PR8-HA VN1203 (R#2) were detected in the lungs or brains. Also in the spleen, the highest virus titres were measured for the wild type VN1203 strain, whereas very low titres were found for PR8 in two animals and in only one ferret for reassortant #2 (Fig. 6C). In nasal turbinates, the VN1203 virus replicated up to titres in the range of 10^7 $TCID_{50}$/ml (Fig. 6D). Reassortant #4 replicated in the nasal turbinates, but to a low extent, whereas PR8 or R#2 was not detected at all in such specimen (Fig. 6D).

None of the subgroup B animals that were infected with the reassortant strains or the parental PR8 died or had to be terminated during the two week observation period. In contrast, one of the three ferrets infected with the VN1203 virus died at day 2 and another animal was sacrificed at day 6 because it had reached the clinical end point of severe neurological disease. In addition, clinical signs of respiratory disease, decrease in activity and neurological symptoms were recorded for the H5N1 wild-type infected animals (data not shown). The two reassortant strains induced less fever than the PR8 donor strain, which in turn induced less fever than the H5N1 virus. Finally, the ferrets infected with the reassortant and PR8 donor strains clearly lost less weight than the H5N1 WT-infected animals (data not shown). Taken together, these results demonstrate a strong attenuation of the two generated H5N1 reassortant viruses in ferrets, as they were not detected in the lung and brain similarly as was shown in parallel for the low pathogenic donor strain PR8. Moreover, the degree of disease induced

by the reassortant strains was comparable to the low pathogenic PR8 strain and clearly attenuated compared to the severe infections caused by the highly pathogenic H5N1 WT virus.

4. Discussion

The rapid generation and large scale production of a matched vaccine virus is a major challenge for global health during a pandemic influenza outbreak to protect individuals against infection with a novel virus strain [20] and [34]. In such a situation it is required to have immediate access to a well growing vaccine virus that incorporates a high amount of the major protective viral antigen HA into its surface membrane. However, experiences in recent years showed that the strategy to produce 6 + 2 reassortants of the PR8 strain may deliver vaccine viruses such as NIBRG-14 with less than average growth and/or antigen yield [18] and [19]. Here, we explored the concept that reverse genetics could be used to generate influenza vaccine seed viruses with improved growth characteristics through modification of the PR8 backbone. To this purpose a set of H5N1-PR8 reassortant influenza viruses with M or PB1 segments derived from avian or human strains were generated and investigated regarding replication in embryonated chicken eggs and HA content. We chose this strategy as the viral M1 protein interacts with surface glycoproteins and contributes to virus assembly [22], whereas the cytoplasmic tail of the M2 protein is required for efficient genome packaging [24] and also contributes to virus assembly [35]. The PB1 gene was included as it was inherited in some circumstances from a seasonal WT isolate or the pandemic H1N1-2009 virus to the corresponding vaccine strains during classical reassortment. Possibly, this was due to an increase in viral polymerase activity [36].

Interestingly, three reassortant viruses in which the M segment of PR8 had been replaced showed an increased HA content compared to NIBRG-14. The M gene of the human Pan99 strain in addition also increased viral growth by more than 2-fold. Importantly, the enrichment in the HA level was maintained after ether/detergent extraction of virions, a common procedure used during split vaccine preparation. The encoded gene products of the Pan99-, R28- and VN1203-M segments are identical to their PR8 homologues in the range of 92.9–95.7% for the M1 protein and 80.6–88.8% for the M2. Some of the variable amino acids in the homologous M1 and M2 proteins of PR8 are specifically found in the Pan99 strain (M1 protein: T167A, R174K, I219V, A227T, A239K; M2 protein: N31S, R54L, Y57H, S82SN, G89S). These strain-specific differences may facilitate a more favourable interaction(s) of the Pan99-M proteins with the cytoplasmic tails of the two H5N1 surface glycoproteins and/or the RNP complex resulting in an increased virus production. However, further mutational analysis will be required to test this hypothesis.

Recently, Rudneva et al. reported that the introduction of the PB1 gene from a low pathogenic avian virus of the H5N2 subtype increased the replication of a corresponding 5 + 2 + 1 PR8 reassortant compared to the conventional 6 + 2 reassortant [37]. Moreover, two reassorted PR8-based vaccine seeds carrying the surface glycoprotein genes of the seasonal Pan99 (H3N2) virus had also received the PB1 gene of the epidemic strain [26]. Our finding that the 6 + 2 reassortant #9, expressing only the HA and NA genes of Pan99, was a poor producer (Fig. 2) supports the conclusion that a matching PB1 gene confers a selective advantage to a corresponding vaccine seed. In case of the engineered H5N1 virus, however, we observed that the inclusion of the VN1203-PB1 gene segment in the 5 + 3 reassortant #7 resulted in a decrease in viral growth. Hence, the contribution of a heterologous PB1 segment to viral replication and antigen yield may depend on the specific strain backbone and/or the species origin of the introduced gene segment and will need to be tested from case to case.

Most reassortant viruses generated for the present study contained the HA and NA proteins of VN1203 instead of the closely related VN1194 used for the generation of NIBRG-14 [17]. These closely related surface proteins differ in only one amino acid in the HA (K52T) and three amino acids in the NA (F54L, N264D, Y324G). Also, the generated recombinants were built on the Mt Sinai-PR8 strain instead of the Cambridge-PR8 backbone [38] and, in principle, those differences could also contribute to the favourable characteristics of the 5 + 2 + 1 reassortant #4. A low influence of the closely related VN1194 and VN1203 glycoprotein genes to vaccine virus growth was recently also noted by Horimoto et al. [39]. Interestingly, in this study an even more pronounced effect on growth enhancement was observed when the HA and NA glycoproteins were incorporated into the background of the PR8-UW (University of Wisconsin) background [39]. The PR8-Mt Sinai strain used in our study differs from PR8-Cambridge in a total of 16 amino acids in all internal gene segments, whereas there are five amino acid variations compared to PR8-UW (data not shown). Further

systematic side-by-side comparisons in terms of virus propagation and possibly also reassortment of the three available PR8 backgrounds may therefore guide a further optimization of vaccine seeds.

In addition to reassortant #4, we measured an improved yield and HA content also for reassortant #2 expressing seven segments of PR8 and the HA gene segment of VN1203. A similar observation was described in a recent study showing that the NA gene segment of the VN1194 strain contributes to the reduced replication and HA quantities of NIBRG-14 [39]. Along the same line, Adamo et al. reported an increase in virus yield in reassortants with chimeric NA protein containing the N-terminus of PR8 and the C-terminus of VN1203 [40]. In our assays we confirmed the recent observation that a PR8 reassortant with the HA/NA genes of VN1203 mimicking our reassortant #3 replicated as well as the parental PR8 [40]. However, this genotype was associated with a strong reduction in virus yield [40] and therefore, we did not follow-up on this reassortant in our analysis. Other recombinants generated during our study such as reassortants #5 and #6 displayed an increased amount of HA antigen, but this was combined with an almost unchanged or decreased replication rate. Thus, the characteristics of reassortant viruses are differentially influenced by distinct gene combinations, the consequences of which cannot be predicted, but have to be determined empirically.

Since it has been described that the M proteins influence virion particle shape [33] selected reassortant viruses were compared with the parental wild type viruses and NIBRG-14 by transmission electron microscopy. It was interesting to note that recombinant PR8 virions had a slightly lower mean circumference value than the H5N1 viruses. The results also indicated that NIBRG-14 and the other investigated viruses formed mainly spherical virion particles. Thus, the reported low antigen yield of NIBRG-14 is not due to a change in virion particle size or morphology. Also, insertions of heterologous M gene segments did not influence the particle size to a large extent. We therefore concluded that the increased surface glycoprotein content of some viruses was not due to an enlarged particle surface, but likely the result from molecular interactions between surface glycoproteins and internal proteins.

Many influenza vaccine production facilities handle PR8-based seed viruses under conditions of the low biosafety level 2. However, alterations in the internal gene composition may possibly alter the low pathogenic character of a PR8-based vaccine virus. Therefore, we compared the virulence of the two reassortant viruses with the highest improvement scores (#2 and #4) to the parental PR8 and the highly pathogenic H5N1 VN1203 strain in the ferret model. Both new reassortant viruses did not replicate in the lungs or brains of ferrets and caused only mild clinical symptoms similar as the vaccine donor strain PR8. Thus, the attenuating features of a monobasic cleavage site in the H5-HA of VN1203 and the low pathogenic strain background of the PR8 virus were maintained in the reassortants. These results indicated that the biosafety profile of these viruses is suitable for normal vaccine production conditions. We noted for PR8 and reassortant #2 that inspite the presence of virus in swab samples from all infected animals on day 3 there was consistently no virus detectable in nasal turbinates. This finding may be conferred by the PR8 neuraminidase gene present in both viruses, but further work will be required to examine this possibility.

All together, the data presented here suggest that antigen yield of reassortant H5N1 influenza vaccine viruses can be improved by insertion of internal gene segments from strains other than the PR8 backbone or the virus providing the glycoprotein genes. Further studies should address the question whether the M gene segments leading to an improved PR8-based H5N1 vaccine seed virus also function in a similar manner in combination with HA and NA gene segments from other virus subtypes. The use of reverse genetics to produce pandemic (or seasonal) influenza vaccines with a gene constellation other than the classical 6 + 2 format could help to produce sufficient amounts of influenza vaccines to protect the population in a short time period against the threat of an influenza pandemic.

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References

[1] Malik Peiris JS. Avian influenza viruses in humans. Revue Scientifique et Technique 2009;28(April (1)):161–73.
[2] Wong SS, Yuen KY. Avian influenza virus infections in humans. Chest 2006;129(January (1)):156–68.
[3] Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. New England Journal of Medicine 2009;360(25):2605–15.
[4] Neumann G, Noda T, Kawaoka Y. Emergence and pandemic potential of swineorigin H1N1 influenza virus. Nature 2009;459(June (7249)):931–9.
[5] 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. The Journal of Experimental Medicine 1960;111(March):387–406.
[6] Beare AS, Kendal AP, Cox NJ, Scholtissek C. Human trials with wild-type H1N1 and recombinant H3N2-H1N1 influenza A viruses of 1977–1978. Infection and Immunity 1980;28(June (3)):753–61.
[7] 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 1975;2(October(7938)):729–32.
[8] Bardiya N, Bae JH. Influenza vaccines: recent advances in production technologies. Applied Microbiology and Biotechnology 2005;67(May (3)):299–305.
[9] Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG. A DNA transfection system for generation of influenza A virus from eight plasmids. Proceedings of the National Academy of Sciences of the United States of America 2000;97(May (11)):6108–13.
[10] Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, et al. Generation of influenza A viruses entirely from cloned cDNAs. Proceedings of theNational Academy of Sciences of the United States of America 1999;96(August(16)):9345–50.
[11] Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A. Rescue of influenza A virus from recombinant DNA. Journal of Virology 1999;73(November (11)):9679–82.
[12] Lina B, Fletcher MA, Valette M, Saliou P, Aymard M. A TritonX-100-split virion influenza vaccine is safe and fulfills the committee for proprietary medicinal products (CPMP) recommendations for the european community for immunogenicity, in children, adults and the elderly. Biologicals 2000;28(2):95–103.
[13] Beyer WE, Palache AM, Osterhaus AD. Comparison of serology and reactogenicity between influenza subunit vaccines and whole virus or split vaccines: a review and meta-analysis of the literature. Clinical Drug Investigation 1998;15(1):1–12.
[14] Bresson JL, Perronne C, Launay O, Gerdil C, Saville M, Wood J, et al. Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial. Lancet 2006;367(May (9523)):1657–64.
[15] Lin J, Zhang J, Dong X, Fang H, Chen J, Su N, et al. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. Lancet 2006;368(September (9540)):991–7.
[16] Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. New England Journal of Medicine 2006;354(March (13)):1343–51.
[17] Nicolson C, Major D, Wood JM, Robertson JS. Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N1 candidate vaccine strain produced under a quality system. Vaccine 2005;23(April (22)):2943–52.
[18] Stephenson I, Gust I, Pervikov Y, Kieny MP. Development of vaccines against influenza H5. The Lancet Infectious Diseases 2006;6(August (8)):458–60.
[19] Harvey R, Wheeler JX, Wallis CL, Robertson JS, Engelhardt OG. Quantitation of haemagglutinin in H5N1 influenza viruses reveals low haemagglutinin content of vaccine virus NIBRG-14 (H5N1). Vaccine 2008;26(December (51)):6550–4.
[20] Partridge J, Kieny MP. Global production of seasonal and pandemic (H1N1) influenza vaccines in 2009–2010 and comparison with previous estimates and global action plan targets. Vaccine 2010;28(July (30)):4709–12.
[21] Bucher D, Popple S, Baer M, Mikhail A, Gong YF, Whitaker C, et al. M protein (M1) of influenza virus: antigenic analysis and intracellular localization with monoclonal antibodies. Journal of Virology 1989;63(September (9)):3622–33.
[22] Ali A, Avalos RT, Ponimaskin E, Nayak DP. Influenza virus assembly: effect of influenza virus glycoproteins on the membrane association of M1 protein. Journal of Virology 2000;74(September (18)):8709–19.
[23] Zhang J, Lamb RA. Characterization of the membrane association of the influenza virus matrix protein in living cells. Virology 1996;225(2):255–66.
[24] McCown MF, Pekosz A. The influenza A virus M2 cytoplasmic tail is required for infectious virus production and efficient genome packaging. Journal of Virology 2005;79(March (6)):3595–605.
[25] McCown MF, Pekosz A. Distinct domains of the influenza A virus M2 protein cytoplasmic tail mediate binding to the M1 protein and facilitate infectious virus production. Journal of Virology 2006;80(August (16)):8178–89.
[26] Ottmann M, Bergeron C, Valette M, Lina B. Genetic content of influenza H3N2 vaccine seeds. PLOS Currents Influenza 2010;August:RRN1165.
[27] Zielecki F, Semmler I, Kalthoff D, Voss D, Mauel S, Gruber AD, et al. Virulence determinants of avian H5N1 influenza A virus in mammalian and avian hosts: the role of the C-terminal ESEV motif in the viral NS1 protein. Journal of Virology 2010;August.
[28] Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. American Journal of Epidemiology 1938;27(May (3)):493–7.
[29] Sawai T, Itoh Y, Ozaki H, Isoda N, Okamoto K, Kashima Y, et al. Induction of cytotoxic T-lymphocyte and antibody responses against highly pathogenic avian influenza virus infection in mice by inoculation of apathogenic H5N1 influenza virus particles inactivated with formalin. Immunology 2008;124(June (2)):155–65.
[30] WHO. Production of pilot lots of inactivated influenza vaccine in response to a pandemic threat: an interim biosafety risk assessment. Releve epidemiologique hebdomadaire/Section d'hygiene du Secretariat de la Societe des Nations = Weekly epidemiological record/Health Section of the Secretariat of the League of Nations 2003;78(November (47)):405–8.
[31] Bourmakina SV, Garcia-Sastre A. Reverse genetics studies on the filamentous morphology of influenza A virus. The Journal of General Virology 2003;84(March (Pt 3)):517–27.
[32] Bourmakina SV, Garcia-Sastre A. The morphology and composition of influenza A virus particles are not affected by low levels of M1 and M2 proteins in infected cells. Journal of Virology 2005;79(June (12)):7926–32.
[33] Roberts PC, Lamb RA, Compans RW. The M1 and M2 proteins of influenza A virus are important determinants in filamentous particle formation. Virology 1998;240(January (1)):127–37.
[34] WHO. Global pandemic influenza action plan to increase vaccine supply; 2006. http://www.who.int/csr/resources/publications/influenza/WHO CDS EPR GIP 2006 1/en/index.html.
[35] Iwatsuki-Horimoto K, Horimoto T, Noda T, Kiso M, Maeda J, Watanabe S, et al. The cytoplasmic tail of the influenza A virus M2 protein plays a role in viral assembly. Journal of Virology 2006;80(June (11)):5233–40.
[36] Wanitchang A, Kramyu J, Jongkaewwattana A. Enhancement of reverse genetics-derived swine-origin H1N1 influenza virus seed vaccine growth by inclusion of indigenous polymerase PB1 protein. Virus Research 2010;147(January(1)):145–8.
[37] Rudneva IA, Timofeeva TA, Shilov AA, Kochergin-Nikitsky KS, Varich NL, Ilyushina NA, et al. Effect of gene constellation and postreassortment amino acid change on the phenotypic features of H5 influenza virus reassortants. Archives of Virology 2007;152(6):1139–45.
[38] Subbarao K, Chen H, Swayne D, Mingay L, Fodor E, Brownlee G, et al. Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. Virology 2003;305(January (1)):192–200. [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 2007;366(September (1)):23–7.
[40] Adamo JE, Liu T, Schmeisser F, Ye Z. Optimizing viral protein yield of influenza virus strain A/Vietnam/1203/2004 by modification of the neuraminidase gene. Journal of Virology 2009;83(May (9)):4023–9.

Tables and Figures

Table 1. Virion dimensions of recombinant viruses.

Virus	Mean area (nm^2)[a]	Mean circumference (nm)[b]
#1	7244 ± 3234	317 ± 69
#2	8308 ± 2328	354 ± 58
#4	8774 ± 2298	357 ± 57
#5	8124 ± 3321	340 ± 74
#8	7809 ± 1315	346 ± 37
#10	8979 ± 3184	373 ± 82

[a] Mean area and standard deviation of randomly selected particles of indicated viruses, determined by electron microscopy (*n* = 100 particles per reassortant).
[b] Mean circumference and standard deviation of randomly selected particles of indicated viruses, determined by electron microscopy (*n* = 100 particles per reassortant).

Table 2. Scoring of the growth capacity and HA/NP ratio of recombinant viruses compared to NIBRG-14.

Virus		HA1/NP ratio[a]	Virus titres[b]	Total score[c]	Ranking
PR8-HA/NA VN 1203-M Pan	R#4	3.2 ± 1.0	2.3	7.4	1
PR8	R#1	1.8 ± 0.8	2.9	5.2	2
PR8-HA VN 1203	R#2	1.8 ± 0.8	2.4	4.1	3
VN 1203-WT-lp	R#10	1.7 ± 0.7	1.7	2.9	4
PR8-HA/NA/M VN 1203	R#6	2.3 ± 0.9	1.2	2.8	5
PR8-HA/NA VN 1203-M R28	R#5	3.7 ± 1.9	0.6	2.2	6
PR8-HA/NA/PB1 VN1203	R#7	1.9 ± 0.8	0.4	0.8	7
NIBRG-14	R#8	1.0 ± 0.0	1.0	1.0	8
PR8-HA/NA Pan	R#9	0.6 ± 0.8	0.6	0.4	9
PR8-HA/NA VN 1203	R#3	n.d.[d]	2.6	n.d.	
VN 1203-WT-hp	R#11	n.d.[d]	0.2	n.d.	

[a] HA/NP ratio *x*-fold to NIBRG-14 (arbitrarily set to 1) and standard deviation, *n* = 4.
[b] Mean EID_{50}/ml *x*-fold to NIBRG-14 (arbitrarily set to 1), *n* = 5.
[c] The total score for each virus is the product of multiplying the relative value for the HA1/NP ratio (third column) with the relative value of virus titre (fourth column) in comparison to NIBRG-14.
[d] Not done.

Figure 1. Gene constellations of generated recombinant/reassortant viruses. The gene constellations of newly generated recombinant/reassortant viruses are shown. White squares indicate gene segments of the vaccine donor strain A/PR/8/34, light grey squares specify gene segments derived from the H5N1-clade 1 strain A/Vietnam/1203/2004 with monobasic cleavage site in the HA gene segment. A light grey oval symbolizes HA gene segment of A/Vietnam/1203/2004 with multibasic cleavage site, black squares indicate gene segments derived from the H5N1-clade 1 strain A/Vietnam/1194/2004. The gene segments of A/chicken/Germany/R28/2003 and A/Panama/2007/1999 are marked with dark grey squares.

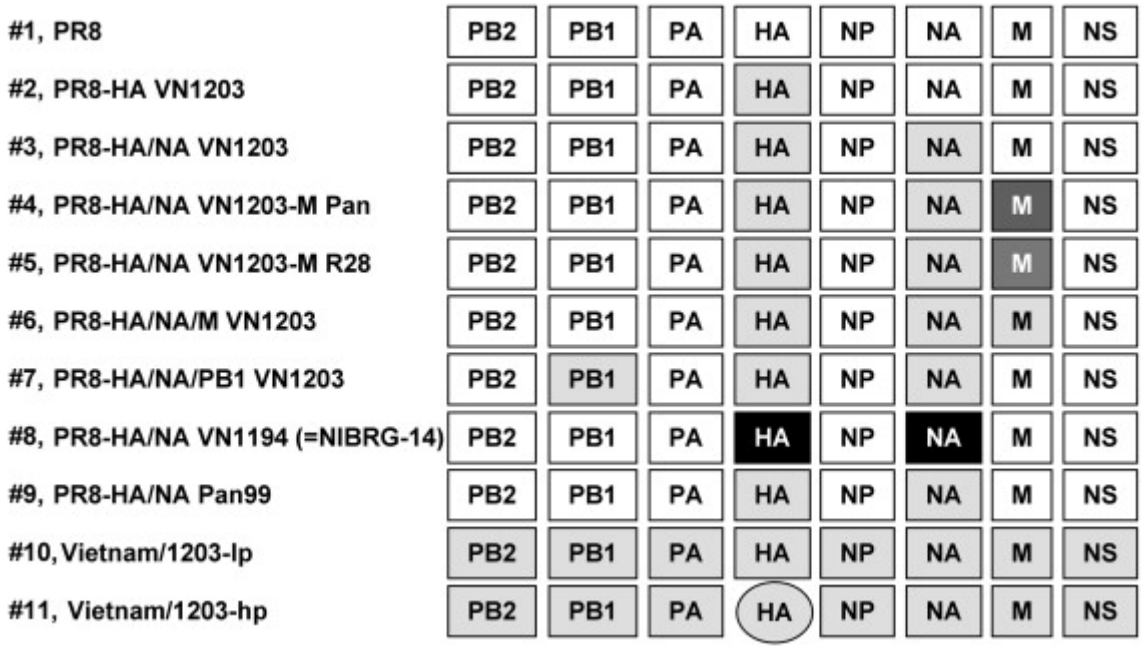

Figure 2. Growth of reassortant/recombinant viruses in 11-day-old embryonated chicken eggs. Embryonated chicken eggs (n = 5 for each virus) were inoculated with 10e3 PFU of the indicated viruses. After inoculation, embryonated chicken eggs were incubated at 37 °C for 48 h. In case of VN1203-hp/-lp incubation time was decreased to 41 h. Harvested allantoic fluid was used for hemagglutination and the EID_{50}/ml values represented by black rhombs were calculated according to the method of Reed and Muench [28]. Black bars specify the mean values from all five eggs. Significant differences for the recombinant viruses # 1 (PR8), #2 (PR8-HA VN1203) and #3 (PR8-HA/NA VN1203) compared to NIBRG-14 are indicated by an asterisk ($p \leq 0.05$).

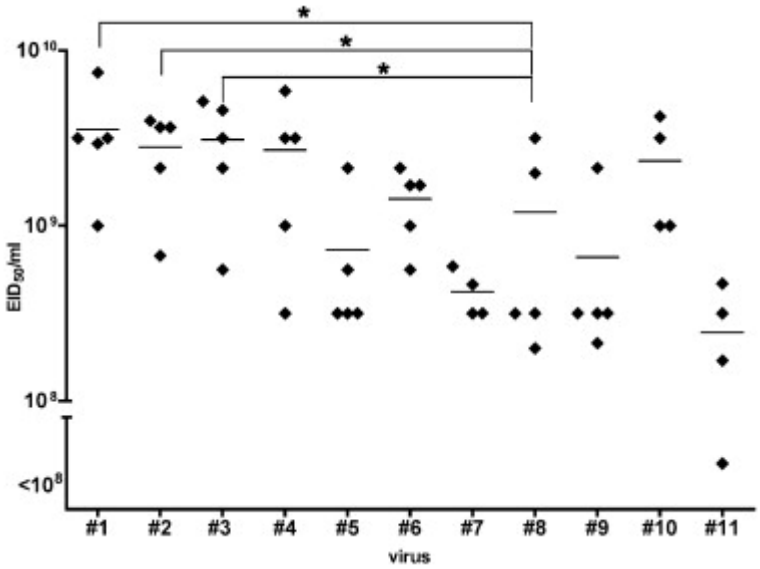

Figure 3. Analysis of HA1/NP ratio in reassortant viruses. Purified virion preparations of indicated recombinant viruses were mock-treated (A) or incubated with PNGaseF (B) prior to separation by SDS gel electrophoresis and protein staining with Coomassie Brilliant blue. (C) Single protein bands were quantified by densitometry and HA1/NP ratios were calculated and set to 1 for NIBRG-14. Mean values of four independent experiments and standard deviations are shown. A significant difference ($p \leq 0.05$) found for the HA1/NP ratio of a virus compared to NIBRG-14 is indicated by an asterisk. (D) Purified virion preparations of the indicated viruses were treated with Tween/ether solution, the surface glycoprotein containing phase was collected, denatured, treated with PNGaseF and proteins were separated by SDS gel electrophoresis. One representative experiment of at least two independent replicates is shown.

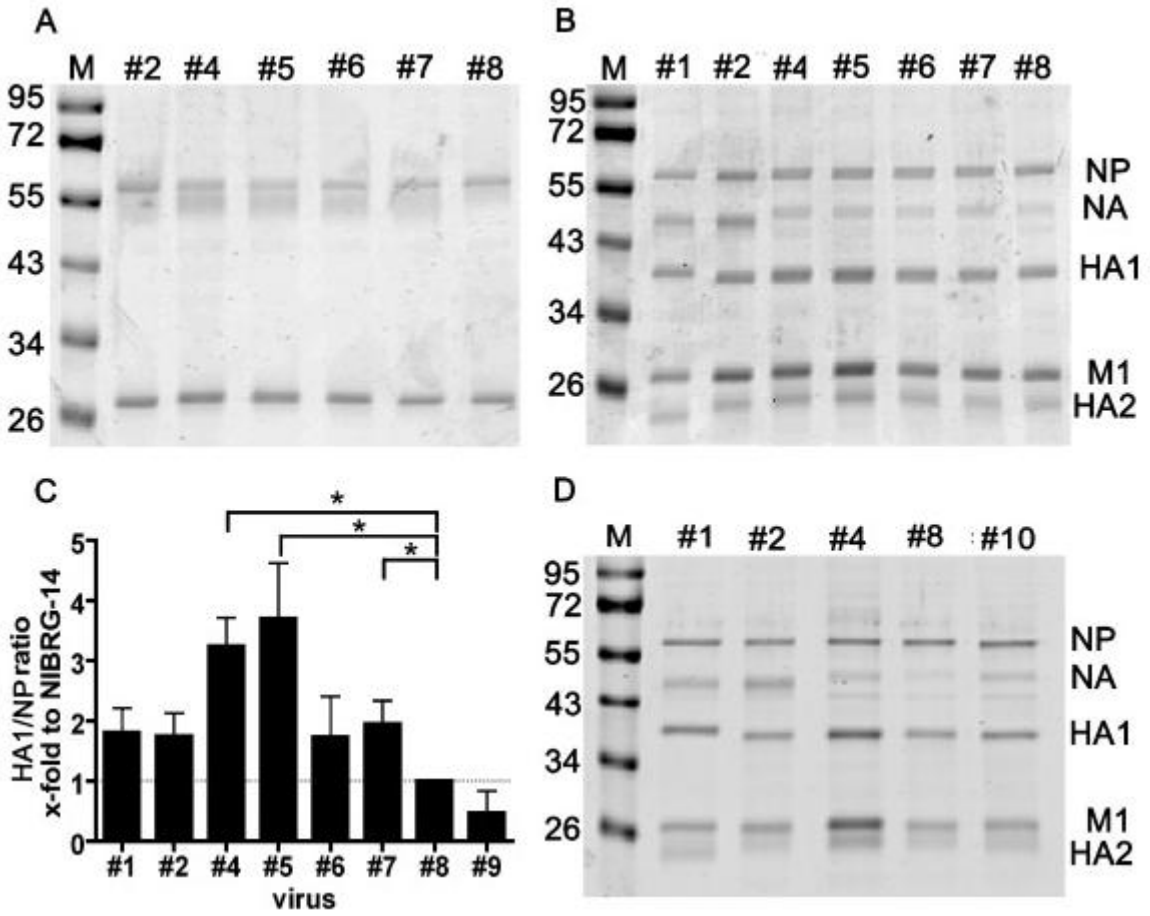

Figure 4. Immuno-negative staining electron microscopy of recombinant and reassortant influenza viruses. HA surface glycoprotein was marked with a H5-HA specific antibody and detected by a secondary antibody coupled to 5 nm colloidal gold (black dots). (A) recombinant A/PR/8/34 (H1N1), (B) A/Vietnam/1203/2004-lp (low pathogenic, H5N1), (C) NIBRG-14 (H5N1), (D) PR8-HA/NA VN1203-M Pan (H5N1). Arrows illustrate immunogold labelled H5-HA surface glycoproteins on the surface of virus particles.

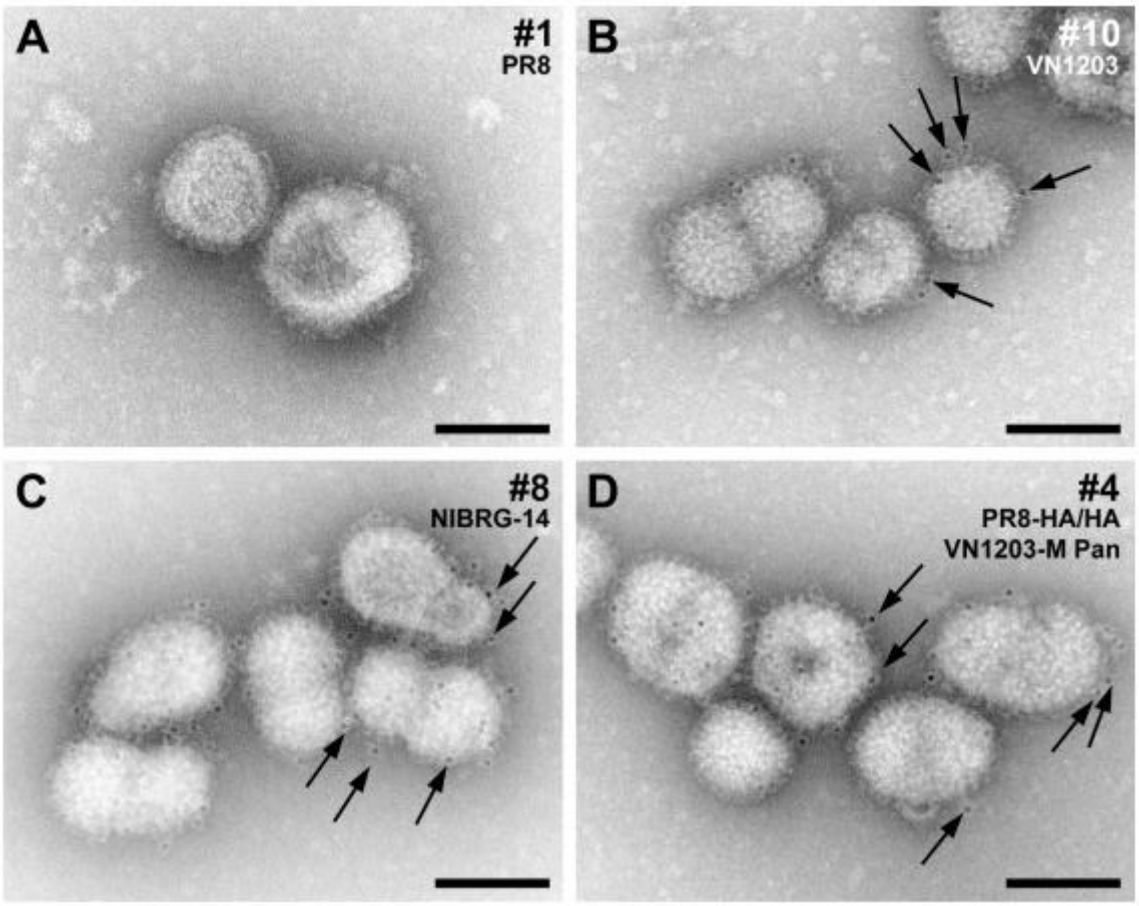

Figure 5. TCID50/ml determination in nasal swabs of infected ferrets. Four groups of ferrets (*n* = 6) were intranasally infected with 0.5 ml containing 10^7 $TCID_{50}$ of either A/PR8/34, PR8-HA VN1203 (R#2), PR8-HA/NA VN1203-M Pan (R#4) or A/Vietnam/1203/2004. On day one to three post infection, nasal swabs were taken and viral loads were assessed by $TCID_{50}$ determination.

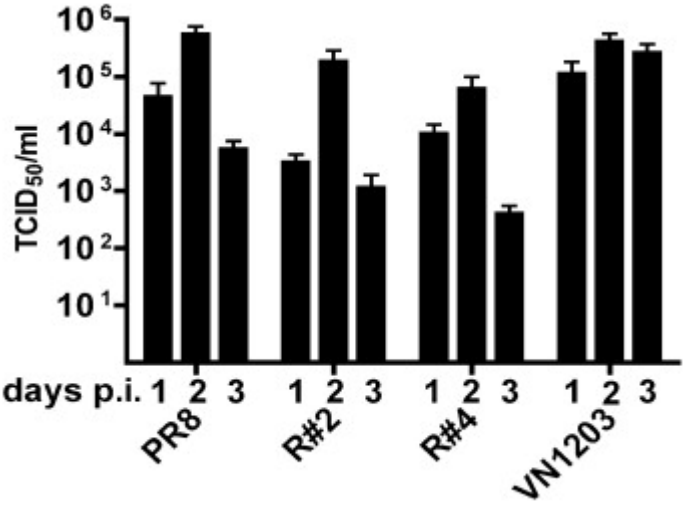

Figure 6. Virological analysis of organ samples from infected ferrets. Four groups of ferrets were intranasally infected with 0.5 ml containing 10^7 $TCID_{50}$ of either A/PR/8/34, PR8-HA VN1203 (R#2), PR8-HA/NA VN1203-M Pan (R#4) or A/Vietnam/1203/2004. Three animals of each group were sacrificed on day 3 and víral loads were titrated by $TCID_{50}$ determination in organ homogenates of the brain (A), lung (B), spleen (C), and nasal turbinates (D). The dotted lines indicate the minimal level of detection.