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Truncation and Sequence Shuffling of Segment 6 Generate Replication-Competent Neuraminidase-Negative Influenza H5N1 Viruses

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Influenza viruses are highly genetically variable and escape from immunogenic pressure by antigenic changes in their surface proteins, referred to as "antigenic drift" and "antigenic shift." To assess the potential genetic plasticity under strong selection pressure, highly pathogenic avian influenza virus (HPAIV) of subtype H5N1 was passaged 50 times in embryonated chicken eggs in the presence of a neutralizing, polyclonal chicken serum. The resulting mutant acquired major alterations in the neuraminidase (NA)-encoding segment. Extensive deletions and rearrangements were detected, in contrast to only 12 amino acid substitutions within all other segments. Interestingly, this new neuraminidase segment resulted from complex sequence shuffling and insertion of a short fragment originating from the PA segment. Characterization of that novel variant revealed a loss of the neuraminidase protein and enzymatic activity, but its replication efficiency remained comparable to that of the wild type. Using reverse genetics, a recombinant virus consisting of the wild-type backbone and the shortened NA segment could be generated; however, generation of this recombinant virus required the polybasic hemagglutinin cleavage site. Two independent repetitions starting with egg passage 30 in the presence of alternative chicken-derived immune sera selected mutants with similar but different large deletions within the NA segment without any neuraminidase activity, indicating a general mechanism. In chicken, these virus variants were avirulent, even though the HPAIV polybasic hemagglutinin cleavage site was still present. Overall, the variants reported here are the first HPAIV H5N1 strains without a functional neuraminidase shown to grow efficiently without any helper factor. These novel HPAIV variants may facilitate future studies shedding light on the role of neuraminidase in virus replication and pathogenicity.

Highly pathogenic avian influenza viruses (HPAIVs) of subtype H5N1 have been circulating in many regions in Asia and Africa for up to 10 years (1), raising concerns of an influenza pandemic.

While wild waterfowl serves as a virus reservoir, poultry—primarily chickens—infected with HPAIV H5N1 succumb to death due to a devastating disease. In addition, the currently used control measures (2), like culling of infected birds, restriction of movement, enforcement of biosecurity, and surveillance, lead to severe economic losses in the poultry industry worldwide. Vaccination against HPAIV H5N1 using inactivated virus preparations was implemented, particularly in developing countries, to combat the disease. However, as influenza A viruses continue to change their antigenicity by antigenic drift, due to base exchanges introduced during the error-prone process of genome replication by the viral polymerase complex, and by antigenic shift, which results from reassortment of genome segments from two viruses (3), vaccines have to be adapted regularly. For application in humans, the World Health Organization (WHO) predetermines the vaccine composition each season. In the veterinary field, nonhomologous vaccines are used, often resulting in nonsterile immunity in the vaccinated poultry flocks and thus a lack of disruption of infection chains. As a consequence, infection of those partially protected birds by circulating recent HPAIV H5N1 leads to the continuous emergence of escape variants (4–6) with an altered antigenic repertoire (6). These viruses are not neutralized by the antibodies present in the vaccinated flocks; hence, the animals are not fully protected, as demonstrated by the reoccurrence of morbidity and mortality (4).

The phenomenon of antigenic escape was classically investigated by the characterization of escape variants generated *in vitro* by virus passaging in the presence of monoclonal antibodies (7, 8). While antigenic sites were thereby successfully identified, such a rather artificial selection is limited to epitope-specific variation only. However, *in silico* analysis of the evolution of both viral surface proteins, i.e., the hemagglutinin (HA) and neuraminidase (NA), revealed several epistatic mutations, highlighting that immunoescape is a polygenic trait (9). In addition, we recently showed that cell culture passaging of HPAIV H5N1 under the selection pressure of a polyclonal chicken-derived serum resulted in attenuated viruses with numerous point mutations in several segments (10). To assess the immunoescape enabled by the considerable genetic plasticity of influenza A viruses under strong, more authentic selection pressure closer to conditions *in vivo*, we passaged an HPAIV H5N1 strain 50 times in the presence of a polyclonal antiserum in embryonated chicken eggs. In contrast to our previous *in vitro* study (10), this experimental approach resulted in replication-competent and stable neuraminidase-negative attenuated H5N1 viruses with large intrasegmental deletions in segment 6 causing a complete loss of neuraminidase activity. Their generation, along with the *in vitro* and *in vivo* features, is the subject of this study.

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MATERIALS AND METHODS

All experiments using HPAIV H5N1 were conducted in biosafety level 3+ containment facilities at the Friedrich-Loeffler-Institut (FLI), Greifswald-Insel Riems, Germany.

Viruses and sera. Ancestor virus for passaging was from the 3rd (egg culture) passage of the reference strain A/Cygnus cygnus/Germany/R65/2006 (H5N1) (11). The initial serum sample (serum sample A) used to implement neutralizing pressure originated from an individual chicken vaccinated twice with a commercial inactivated vaccine of the H5N2 subtype (Nobilis Influenza H5N2; Intervet, Unterschleißheim, Germany) and afterwards boosted by use of a challenge infection with HPAIV H5N1 R65/p17 (a passaged but highly related variant of the original R65 strain). This immunization procedure was selected to allow the development of a maximum of serum antibodies against immunogenic influenza virus proteins, which would enable efficient immunogenic pressure on HPAIV H5N1 (immunization schedule data are available upon request). The immune serum from another chicken (serum sample B) also vaccinated twice with the inactivated H5N2 vaccine and challenged with HPAIV R65/p17 was used as a second test serum sample both for the neutralization test and for passaging the 30th passage of H5N1 R65 in the repetition experiment generating the second escape variant virus, EscEgg50B. A third serum sample (serum sample C) originating from a chicken vaccinated once with the commercial inactivated H5N2 vaccine and afterwards challenged with the original HPAIV A/Swan/Germany/R65/2006 (H5N1) (R65/06) was used for the generation of the third escape variant virus, EscEgg50C. All three serum samples were further characterized using the hemagglutination inhibition (HI) test (see below) against the ancestor virus H5N1 R65, which scored with the same HI titer of 1:128. The HI titers of the three serum samples were comparable to the titers reported for similar experiments (6) and also to the HI titer for chicken serum from an evaluation study performed under field conditions using the H5N2 vaccine and HPAIV H5N1 R65 challenge infection (12). Furthermore, serum samples B and C (serum sample A was completely used for passaging, neutralization testing, and the HI assay and was therefore no longer available) were tested using a commercial enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against the N1 protein (ID Screen influenza virus N1 antibody competition ELISA kit; ID-vet, Montpellier, France). Both serum samples scored negative in the N1 ELISA.

In addition, serum samples B and C as well as a negative commercially available chicken serum sample (Sigma-Aldrich) were tested at dilutions ranging from 1:2 to 1:512 against a defined amount of ancestor virus H5N1 strain R65 using a neuraminidase activity test (see below). Interestingly, there was an inhibitory effect on the neuraminidase activity detectable in the antibody-positive serum that was dilutable, and values recorded for the nonimmunized control chicken serum sample were statistically significantly different from the data collected for immune serum samples B and C (Student's *t* test; data not shown).

Passaging in egg culture. The principle steps for passaging of virus under positive serum pressure were as follows. Virus was incubated with eight different antiserum dilutions at room temperature in 0.2 ml Dulbecco modified Eagle medium supplemented with 5% fetal calf serum for 1 h with gentle agitation. Subsequently, eight embryonated specific-pathogen-free (SPF) chicken eggs (10 days old) were each inoculated with one of the antiserum-incubated virus preparations via the allantoic cavity and checked daily for embryonic death. Five days after inoculation, allantois fluid was harvested and MDCK cells (RIE1061; Collection of Cell Lines in Veterinary Medicine, FLI, Greifswald-Insel Riems, Germany) were inoculated with 50 μl of the allantois fluid. After incubation of the cells for 3 days, the cytopathic effect was assessed via light microscopy. The virus from that allantois fluid specimen with the maximum amount of serum still allowing viral growth was chosen for the next egg passage.

In total, 50 egg passages using a single polyclonal antiserum (serum sample A) were done, resulting in the virus EscEgg50A (A/hen's egg/Germany/[A/Cygnus cygnus/Germany/R65/2006]-EscEgg50-escape/2009 [H5N1]). A control virus was mock passaged without serum in egg culture 50 times in parallel (CoEgg50). Starting with the 30th passage of the experiment, two additional distinct escape variants (EscEgg50B, EscEgg50C) were generated by passaging in egg culture (until 50 passages were achieved) in the presence of two different polyclonal serum samples from chickens (serum samples B and C).

Whole-genome sequencing. EscEgg50A and CoEgg50 were sequenced with a Genome Sequencer FLX (GS FLX) instrument (Roche, Mannheim, Germany) according to the protocol of Höper and coworkers (13) with the modifications of Leifer and colleagues (14). In addition, EscEgg50A was sequenced after preparation of a randomly primed cDNA sequencing library for Titanium sequencing with the GS FLX instrument according to the manufacturer's protocol. Moreover, after reverse transcription-PCR (RT-PCR) amplification of segment 6 of the ancestor virus, EscEgg50A, and viruses from intermediate passages (primer sequences are available upon request), DNAs were sequenced with the Genome Sequencer FLX instrument according to the manufacturer's recommendations. Raw data were analyzed using software provided with the Genome Sequencer FLX instrument. In addition, the NA segments of EscEgg50A, EscEgg50B, and EscEgg50C and the HA segment of the recombinant escape variant EscEgg50Arec were sequenced using classical Sanger sequencing (15). Moreover, the 3′ and 5′ termini of EscEgg50A segment 6 were determined by classical Sanger sequencing after rapid amplification of cDNA ends (RACE; 3′ and 5′ RACE system for rapid amplification of cDNA ends; Invitrogen, Darmstadt, Germany).

The rearrangement of EscEgg50A, -B, and -C segment 6 was analyzed using the Mauve (v 2.3.1.) program (16) with the MauveAligner algorithm. The coordinates of the rearrangements were then used for plotting the rearrangement graph in R (17).

Generation of recombinant viruses. The ancestor virus R65/06 and the mutants were generated by previously described reverse genetics techniques (18, 19). To obtain a plasmid encoding the EscEgg50A NA, we performed target-primed plasmid amplification using the pHW2000-R65NP plasmid (18) and the full-length PCR amplicon.

We constructed a pHW2000 EscEgg50A NA-deletion plasmid by mutating the start codon ATG (nucleotides [nt] 21 to 23) to ACG (the primer sequence is available upon request). Furthermore, we generated a pHW2000 plasmid expressing a minimal segment 6 and enhanced green fluorescent protein (EGFP), with the plasmid carrying the first 118 nucleotides from segment 6 (from EscEgg50C) upstream of the EGFP-encoding sequence, followed by the 77 terminal nucleotides from segment 6 (from EscEgg50A). The four ATG triplets within the first 118 nucleotides were silently mutated, resulting in a first start codon at position 116 for expression of the EGFP protein.

Neutralization assay. The virus neutralization test (VNT) was performed according to a previously described procedure (21) with a few modifications. In brief, serum samples were heat inactivated for 30 min at 56°C, and 3-fold serial dilutions were prepared in a 50-μl volume of cell culture medium in 96-well plates. The diluted serum samples were mixed with an equal volume of medium containing strain R65/06, the escape mutant EscEgg50A, or the passaged control virus CoEgg50 at a concentration of 10^2 50% tissue culture infective doses ($TCID_{50}$s)/well. After 1 h of incubation at 37°C in a 5% CO_2 humidified atmosphere, 100 μl of MDCK cells at 1.5×10^5/ml was added to each well. The plates were incubated for 3 days at 37°C in 5% CO_2. Viral replication was assessed by visually scoring the cytopathic effect without staining. Each assay was validated by comparison with positive- and negative-control sera from chicken and by titration of the virus dilutions used. Results were statistically evaluated by using a one-way analysis of variance (ANOVA).

Viral growth kinetics and plaque size measurement. Growth kinetics were assessed on MDCK cells by infecting the cells at a multiplicity of infection (MOI) of 1 or 0.01. At the indicated times after infection, intra- and extracellular virus titers were determined.

To examine virus-induced plaque sizes, MDCK cells were seeded in six-well plates (Nunc; Thermo Fisher Scientific, Langenselbold, Germany) and infected with R65/06, EscEgg50A, and EscEgg50Arec using an

MOI of 0.01. Twenty-four hours after infection under an agarose overlay, the plaque diameters of 50 randomly selected plaques of each virus were determined (after fixation and staining against nucleoprotein [NP]; see below), and mean diameters and standard errors were calculated. Values for parental strain R65/06 were set to 100%, and the plaque diameters observed for the mutant viruses were expressed relative to this value.

Viral growth analysis after supplementation of bacterial sialidase was performed on MDCK cells incubated for 2 h in the presence of 1 U *Clostridium perfringens* neuraminidase (Roche Diagnostics, Mannheim, Germany) per ml. Afterwards, the cell culture was infected at an MOI of 0.1, still in the presence of the bacterial sialidase. The viral titers of the supernatants were determined after 24 h and 48 h of incubation. Analysis of variance by the Kruskal-Wallis test was used to determine the statistical relevance of the collected data.

Hemagglutination assay. Hemagglutination activity was determined in microtiter plates by using 0.5% chicken erythrocytes. The reactions were performed in phosphate-buffered saline (PBS) at room temperature (approximately 20°C).

Hemagglutination inhibition assay. The hemagglutination inhibition assay was conducted as described in the International Office of Epizootics (OIE) *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (22).

Indirect immunofluorescence assay. MDCK cells grown on coverslips in 4-well culture plates (Lab-Tek chamber slide system; Nunc; Thermo Fisher Scientific, Langenselbold, Germany) were infected with EscEgg50A virus or the ancestor virus R65/06 and incubated for 72 h. Subsequently, the cells were fixed with methanol-acetone (1:1) for 30 min. For the detection of NA and NP, the fixed cells on the coverslips were incubated in the given order with each of the following monoclonal antibodies for 60 min at room temperature: (i) mouse anti-N1 monoclonal antibody (N1 18.2.5; Malte Dauber, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany) diluted 1:5 in PBS, (ii) Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, Life Technologies, Darmstadt, Germany) diluted 1:1,000 in PBS as a secondary antibody, (iii) anti-NP monoclonal antibody (ATCC, HB-65) diluted 1:20 in PBS, and (iv) Alexa Fluor 546 donkey anti-mouse IgG secondary antibody (Invitrogen) diluted 1:1,000 in PBS. Fluorescence was detected using an Axioskop microscope (Zeiss, Jena, Germany).

Western blot analysis. Forty-eight hours after infection, MDCK cells infected with EscEgg50A or the ancestor virus R65/06 were lysed by a freeze-thaw procedure in extraction buffer (1% Triton X-100, 2 mM EDTA, 0.15 M NaCl, 20 mM Na_2HPO_4, pH 7.6) containing proteinase inhibitor (Complete Mini; Roche Diagnostics, Mannheim, Germany). The resulting protein extracts and PageRuler prestained protein ladder (Thermo Fisher Scientific) were separated on a 10% SDS-polyacrylamide gel under reducing conditions and transferred to nitrocellulose membranes (Whatman, Dassel, Germany) by the wet Western methodology. After blocking overnight in Tris-buffered saline supplemented with 0.1% Tween (TBS-T) and 5% skim milk, the blot was incubated with the anti-HA antibody (23) diluted 1:5,000 in TBS-T or the anti-N1 antibody (23) diluted 1: 5,000 in TBS-T. As secondary antibody, a horseradish peroxidase-conjugated antimouse antibody (Dianova, Hamburg, Germany) diluted 1:20,000 in TBS-T was used. Antibody binding was visualized by chemiluminescence (Supersignal West Pico chemiluminescence kit; Pierce, Bonn, Germany) using a ChemoCam system (Intas, Göttingen, Germany).

RT-qPCR assays. RNA extraction from allantois fluid was performed using a QIAamp viral RNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The NA truncation-specific reverse transcription real-time quantitative PCR (RT-qPCR) assay was optimized to work with a probe complementary to an ancestor sequence maintained within the EscEgg50A segment 6 sequence and EscEgg50A virus segment 6-specific primers (sequences are available upon request). In addition, H5- and N1-specific sequences were detected in a duplex RT-qPCR (24). A heterologous internal control system (25) was detected as an extraction control. The EscEgg50A-specific RT-qPCR assay was carried out using an AgPath-ID one-step RT-PCR kit (Ambion; Applied Biosystems, Life Technologies, Darmstadt, Germany). The total reaction volume of 25 μl contained 4 μl RNase-free water, 12.5 μl 2× AgPath-ID one-step RT-PCR master mix, 1 μl AgPath-ID one-step RT-PCR enzyme mix, 1.25 μl an NA-specific 6-carboxyfluorescein-labeled primer-probe mix, 1.25 μl an extraction control-specific 4,4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein-labeled primer-probe mix, and finally, 5 μl RNA template. The assay was run on an ABI 7500 real-time PCR system (Applied Biosystems, Life Technologies, Darmstadt, Germany). The following thermal profile was used: reverse transcription at 45°C for 10 min, a PCR initial activation step at 95°C for 10 min, and 42 cycles of three-step cycling consisting of denaturation at 95°C for 15 s, annealing at 55°C for 20 s, and extension at 72°C for 30 s.

NA activity assay. The ancestor virus R65, the control virus CoEgg50, the escape variant EscEgg50A, and the recombinant escape variant EscEgg50Arec (see below) were inactivated using 1.5 mM binary ethyleneimine (20) before downstream processing. All virus preparations were adjusted to the same genome load quantified by RT-qPCR analysis of viral segment 7 (26). Subsequently, the neuraminidase activity was measured using an NA-XTD influenza virus neuraminidase assay kit (Applied Biosystems) according to the manufacturer's instructions. The luminescence was determined on a Tecan Infinite 200 instrument (Tecan, Crailsheim, Germany). The neuraminidase activity was examined with or without applying the neuraminidase inhibitor oseltamivir carboxylate (10.56 nM, 256 nM, and 6,600 nM; Hoffmann-La Roche Inc., Nutley, NJ). Except for the neuraminidase activity of recombinant virus EscEgg50Arec, neuraminidase activity was determined from two independent virus preparations in each of four replicates. For EscEgg50Arec, neuraminidase activity was measured in only a single virus preparation in four replicates for each inhibitor concentration. A no-virus control was included for every inhibitor concentration. After normalization of the raw data, the signal intensities were divided by the average signal for the respective no-virus control to get adjusted values for every inhibitor-virus combination. For comparison, the reference viruses A/Mississippi/3/2001 (H1N1) wild type (274H) and the neuraminidase inhibitor-resistant A/Mississippi/3/2001 (H1N1) mutant (274Y), kindly provided by the Neuraminidase Inhibitor Susceptibility Network (NISN), were included.

Animal experiments. The animal trials were approved by the state ethics commitee of the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei, Mecklenburg-Vorpommern, under registration number LVL MV/TSD/7221.3-1.1-003/07.

IVPI. The determination of the intravenous pathogenicity index (IVPI) according to the OIE standard protocol (22) is the appropriate test to assess the pathogenicity of a certain influenza virus strain in avian species. The IVPI indicates the mean clinical score of 10 6-week-old chickens after intravenous inoculation. Thereto, groups of 10 SPF chickens (Lohmann Tierzucht, Cuxhaven, Germany) were intravenously infected with the indicated viruses at $10^{4.5}$ $TCID_{50}$s/animal. Birds were scored on a scale ranging from 0 to 3, with 0 being healthy, 1 being sick, 2 being severely sick, and 3 being dead. After 10 days of evaluation, the IVPI was calculated as the average score of 10 birds on 10 days. Viruses are classified as highly pathogenic if their IVPI is 1.2 or higher (22).

Oronasal infection. Ten SPF chickens were housed together and infected with the indicated viruses by oronasal application of $10^{4.5}$ $TCID_{50}$s/animal. Another group of 10 SPF chickens was infected oronasally with $10^{4.5}$ $TCID_{50}$s/animal of the control virus, CoEgg50. The chickens were checked daily for clinical symptoms, and 14 days after inoculation, serum samples were taken from individual chickens. Thereafter, surviving chickens were challenged by oronasal application of 10^6 $TCID_{50}$s/animal of the ancestor virus R65/06. Serum samples were taken from surviving animals at 9 days postchallenge.

Serological analysis. Preexperimental serum samples from individual chickens and samples from surviving animals were heat inactivated at 56°C for 30 min and examined for the presence of antibodies against the

nucleoprotein of avian influenza A virus (ID Screen influenza A virus antibody competition ELISA kit; ID-vet, Montpellier, France), antibodies against the H5 protein (ID Screen influenza virus H5 antibody competition ELISA kit; ID-vet), and finally, antibodies against the N1 protein (ID Screen influenza virus N1 antibody competition ELISA kit; ID-vet). Sera were also tested by an *in vitro* neutralization assay (described above) using the ancestor virus R65/06 to be neutralized.

Nucleotide sequence accession numbers. All genome segments of EscEgg50A were sequenced and deposited in the GISAID EpiFlu database (www.gisaid.org) under accession numbers EPI338332 to EPI338339. CoEgg50 was deposited under accession numbers EPI338340 to EPI338347, EscEgg50B segment 6 under EPI383000, and EscEgg50C segment 6 under EPI383001.

RESULTS

To simulate the selection and emergence of an escape H5 virus in a vaccinated flock of chickens, an H5N1 virus was forced to replicate under the multifarious immunogenic pressure applied by a polyclonal chicken serum. The ancestor virus HPAIV A/Swan/Germany/R65/2006 (H5N1) (R65/06) (11) was passaged 50 times in egg culture in the presence of a polyclonal chicken serum (serum sample A), resulting in the escape variant virus EscEgg50A. A control virus that was mock passaged 50 times in egg culture without serum was designated CoEgg50.

EscEgg50A is a novel H5N1 variant with a unique truncation and sequence shuffling within segment 6 including intersegmental recombination. Expecting several individual amino acid substitutions, all genome segments of EscEgg50A were sequenced and deposited in GISAID (accession numbers EPI338332 to EPI338339). Twelve individual amino acid substitutions within the whole genome (Table 1) were detected for EscEgg50A, while 8 amino acid (aa) substitutions were found for the CoEgg50 control virus (Table 1). For both viruses—EscEgg50A and CoEgg50—the amino acid substitutions were scattered across the genome. Interestingly, the EscEgg50A HA protein acquired only a single amino acid exchange (E385K) during 50 passages, while an identical number of passages without immune serum pressure led to 3 aa substitutions within the HA of the CoEgg50 control virus (Table 1). Besides the aforementioned amino acid substitutions, EscEgg50A carried a prominent sequence variation within segment 6 in which major parts of the coding region were deleted and the remaining parts were shuffled (Fig. 1A; Table 2). In addition, 29 bases from PA-coding segment 3 were incorporated into segment 6. In total, segment 6 of EscEgg50A had a length of 680 nt with one single open reading frame from nt 21 to nt 209 (62 aa) encoding the 51 N-terminal amino acids of the original NA protein followed by 11 additional unrelated amino acids. To rule out the possibility that sequences were shuffled to regions not covered by the amplification product, the sequence was confirmed using the standard random RNA sequencing protocol for the GS FLX instrument. In addition, RACE PCRs were performed to sequence the termini of segment 6. The terminal sequences determined did not differ from the ancestor segment terminal sequence.

EscEgg50A is negative for the NA protein and for NA activity. Indirect immunofluorescence staining and Western blotting using monoclonal as well as polyclonal NA-specific antibodies were used to confirm the loss of the NA protein. In cell cultures infected with EscEgg50A, no fluorescence was detected after staining with a monoclonal antibody against NA (Fig. 2). The cell cultures were costained with anti-NP antibodies, resulting in fluorescence showing the presence of nucleoprotein in the EscEgg50A-infected cell cultures (Fig. 2). Western blot analysis using a polyclonal anti-NA serum demonstrated a distinct band at 56 kDa in the protein preparation of the ancestor virus R65/06. This band was absent from the EscEgg50A virus preparation (Fig. 3A). Protein detection using a polyclonal anti-HA serum confirmed the presence of viral protein in the protein preparations used (Fig. 3B).

To verify the loss of the enzymatic function, a neuraminidase activity assay was performed. The ancestor virus R65/06, the control virus CoEgg50, the EscEgg50A virus, the recombinant EscEgg50Arec virus, and two reference viruses of subtype H1N1 were tested in parallel. Neuraminidase activity was determined with or without addition of the neuraminidase inhibitor oseltamivir. Independently of the inhibitor concentration, the EscEgg50A and EscEgg50Arec preparations with NA deletions showed no neuraminidase activity at all (Fig. 4), while the reference viruses as well as R65/06 and CoEgg50 exhibited neuraminidase activities, as anticipated (Fig. 4). Taken together, these results demonstrate the loss of neuraminidase enzyme activity of EscEgg50A and the recombinant EscEgg50Arec.

The replication competence of EscEgg50A does not require any compensatory mutations or a functional NA protein but does require a polybasic HA cleavage site. A reverse genetics sys-

TABLE 1 Amino acid substitutions acquired by viruses after 50 passages in egg culture with (EscEgg50A) or without (CoEgg50) use of a polyclonal chicken antiserum

Protein	Amino acid position	Amino acid residue[a]		
		R65/06	EscEgg50A	CoEgg50
PB2	368	Q	Q	K
	700	E	K	E
PB1	738	E	E	G
PB1F2	56	A	V	A
	77	L	W	L
PA	208	T	I	T
	595	M	I	M
HA	143	A	A	V
	210	P	P	S
	313	L	L	H
	385	E	K	E
NP	351	R	K	R
NA			Deletions and rearrangement	None
M1	26	Q	Q	R
	50	P	P	T
	88	G	G	R
	125	A	T	A
	175	H	Q	H
M2	14	E	A	E
	68	V	A	V
NS1			None	None
NS2	22	A	E	A

[a] For comparison, the amino acids of ancestor strain R65/06 are given.

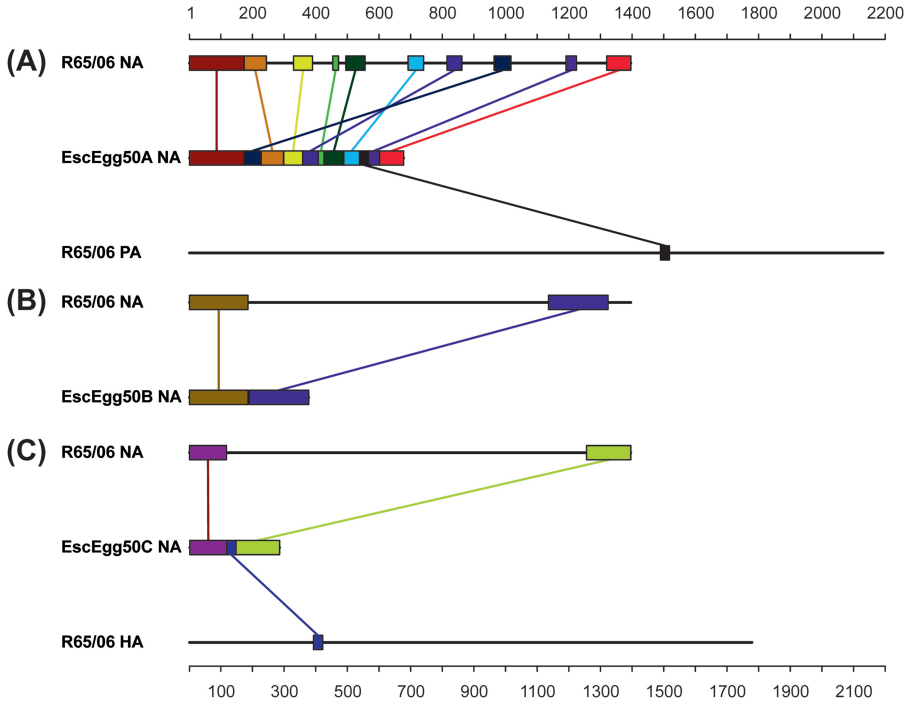

FIG 1 Nucleotide sequence rearrangements in segment 6 of virus variants EscEgg50A, EscEgg50B, and EscEgg50C. (A) Rearranged EscEgg50A segment 6 together with R65/06 donor segments 6 and 3; (B) rearranged EscEgg50B NA with donor segment 6; (C) rearranged EscEgg50C NA with donor segments 6 and 4. The differently colored rectangles depict the different portions of the donor segments that were rearranged in the EscEgg50A, EscEgg50B, and EscEgg50C variants. Those parts of the donor segments that were not incorporated are depicted as black lines. The numbers on the scales at the top and bottom represent nucleotide positions.

tem of HPAIV H5N1 strain R65/06 (18) was used to analyze the role of potential compensatory mutations. While a virus composed of only seven segments could never be generated, the ancestor virus containing R65/06 segments HA, NP, M, NS1, PA, PB1, and PB2 together with the EscEgg50A NA gene was readily reconstituted (EscEgg50Arec). Furthermore, the measurement of its neuraminidase activity demonstrated the loss of a functional N1 protein (Fig. 4). Therefore, none of the 12 additional amino acid variations within the other segments of EscEgg50A were necessary to enable replication and growth in the absence of a functional neuraminidase; however, they did assist with viral replication (Fig. 5). Moreover, we constructed a recombinant virus consisting of 7 segments (HA, NP, M, NS1, PA, PB1, PB2) from the ancestor R65/06 strain combined with the EscEgg50A segment NA^{ATG-}, in which the original ATG start codon was silenced. The full replication competence of the NA^{ATG-} virus in cell culture demonstrated the nonessential character of the remaining coding information of

TABLE 2 Source sequences of the different EscEgg50 segment 6 variants

Position within mutant virus strain			Position within ancestor virus R65/06			
Variant	First nucleotide	Last nucleotide	Segment	First nucleotide	Last nucleotide	Sequence identity[a]
EscEgg50A	1	173	6	1	173	172/173
	174	227	6	964	1017	54/54
	228	298	6	174	244	70/71
	299	359	6	330	390	61/61
	360	407	6	815	862	48/48
	408	426	6	454	472	17/19
	427	488	6	495	556	62/62
	489	538	6	692	741	51/51
	539	567	3	1490	1518	29/29
	568	601	6	1191	1224	34/34
	602	678	6	1320	1396	75/77
EscEgg50B	1	186	6	1	186	186/186
	190	378	6	1136	1324	188/189
EscEgg50C	2	119	6	1	118	118/118
	119	148	4	393	422	30/30
	146	286	6	1256	1396	140/141

[a] Data represent the number of nucleotides that were identical/total number of nucleotides in the sequence.

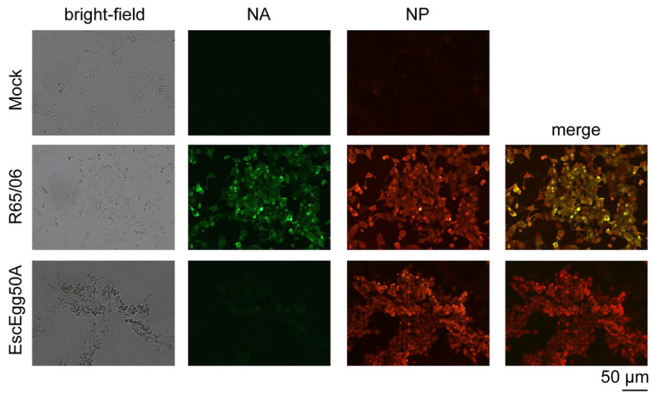

FIG 2 Microscopic analysis of MDCK cells infected with ancestor virus R65/06 or EscEgg50A. For immunofluorescence, cells were stained at 48 h postinfection with monoclonal antibodies specific for NA or NP.

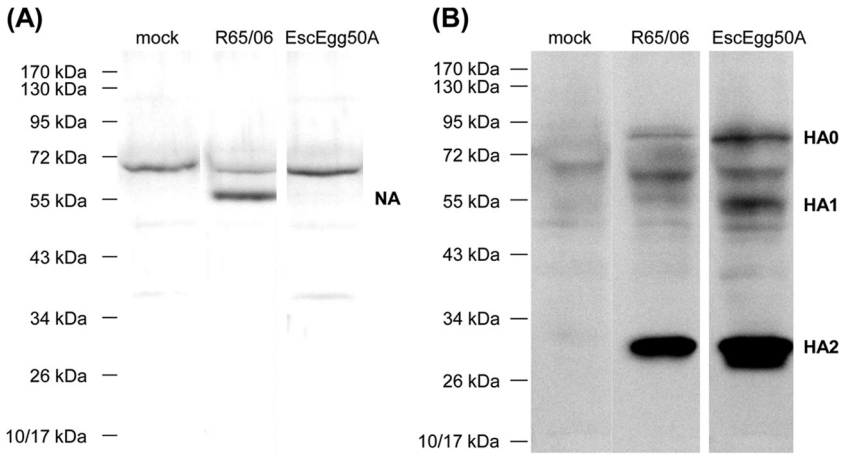

FIG 3 Detection of the hemagglutinin and neuraminidase proteins by Western blotting. Cells were infected with EscEgg50A or ancestor virus R65/06, and lysates for Western blot analysis were collected at 48 h postinfection. Both gels were loaded with equal protein amounts and run under equal conditions. The molecular masses of the marker proteins are indicated. (A) Detection of the neuraminidase protein; (B) detection of the hemagglutinin precursor HA0 and its processing products, HA1 and HA2.

the truncated segment 6 within EscEgg50A. Furthermore, a recombinant virus expressing EGFP from a minimal segment 6 (118 N-terminal nucleotides and 77 C-terminal nucleotides) was generated (data not shown), confirming the results observed with the NA^{ATG-} virus. In order to rule out the possibility that compensatory mutations occurred within HA after transfection, the HA sequence of the rescued EscEgg50Arec virus was determined by classical Sanger sequencing. The HA sequence (nt 9 to 1704) of the rescued EscEgg50Arec virus was identical to the HA sequence of ancestor virus R65/06. It has previously been shown for the same H5N1 strain that exchanging the polybasic HA cleavage site motif RRRKK(R/G) for the monobasic motif ET(R/G) (but with the strain possessing a competent NA) resulted in nearly identical multistep titers in the supernatant of MDCK cells in the presence of trypsin (27). Therefore, the ability to replicate and spread from infected cells is not impaired, as long as trypsin is provided.

To evaluate the impact of the polybasic HA cleavage site, we generated a recombinant virus which carried the EscEgg50A NA gene together with a monobasic HA cleavage site. Because an NA-negative monobasic HA virus could not be generated, we suggest

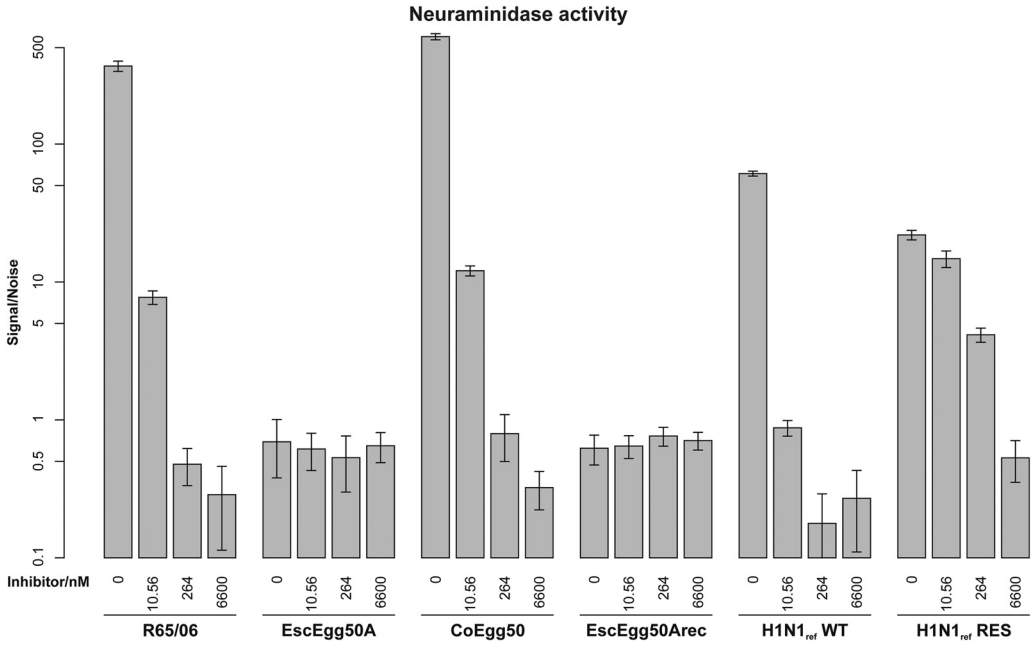

FIG 4 Measurement of neuraminidase activity. Preparations of R65/06, CoEgg50, EscEgg50A, and EscEgg50rec viruses and two reference (ref) viruses of the H1N1 subtype were adjusted to an equal genome load and tested using an NA-XTD influenza virus neuraminidase assay kit. Three different concentrations of the neuraminidase inhibitor oseltamivir carboxylate were used. Signal/noise values were determined from two biological replicates with four technical replicates each for all viruses except EscEgg50rec, for which four technical replicates from one biological replicate were used. Error bars indicate standard deviations. WT, wild type; RES, resistant.

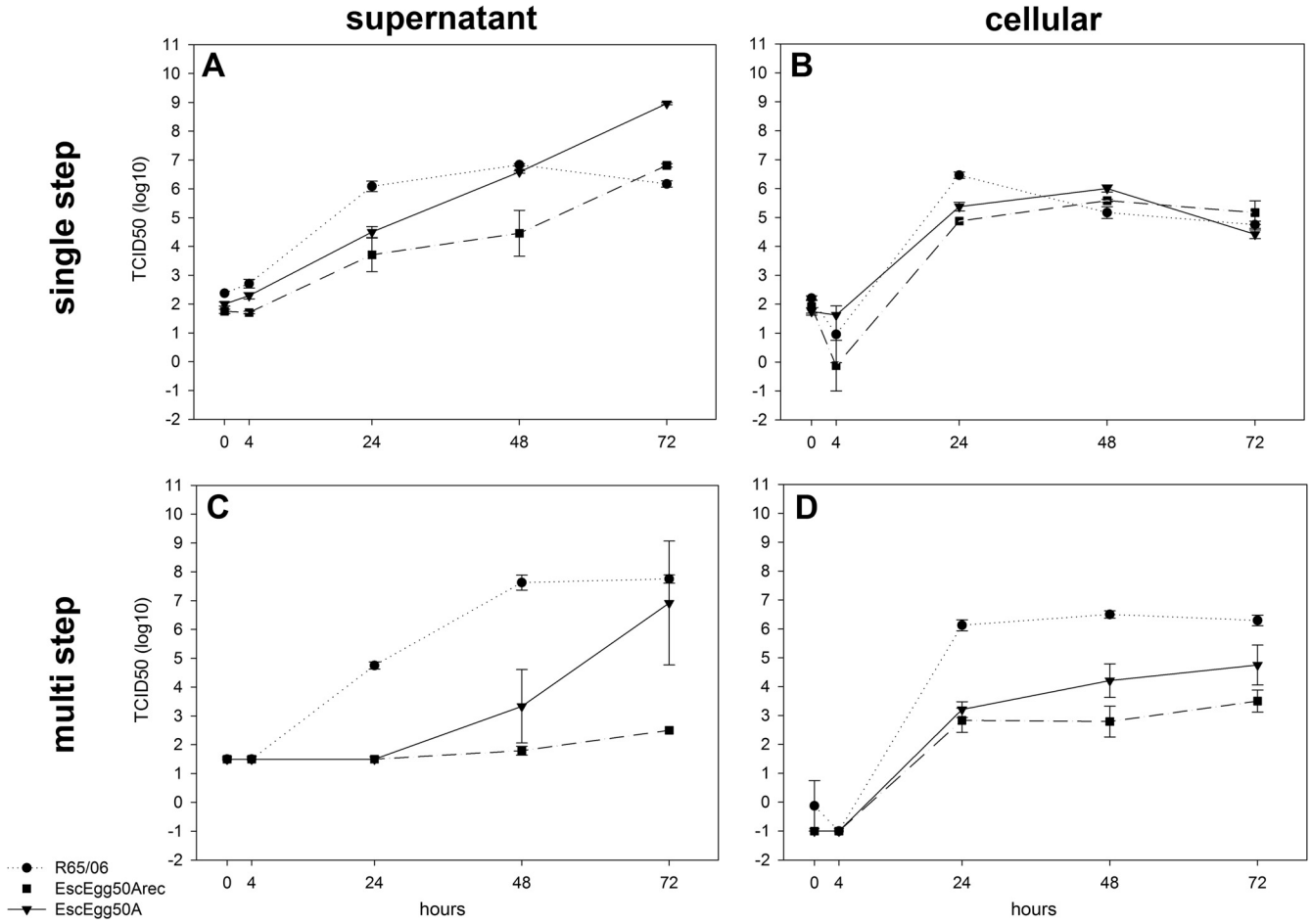

FIG 5 Growth properties of R65/06, EscEgg50A, and EscEgg50Arec. For single-step growth kinetics, MDCK cells were infected at an MOI of 1 (A, B); for multistep analysis, an MOI of 0.01 was used (C, D). Cell culture supernatant (extracellular; A, C) and cellular (cell-associated; B, D) fractions were collected at the indicated time points. Viral titers were determined by titration. The results are mean values of three independent experiments. Error bars indicate SEMs.

that a polybasic HA protein may be a prerequisite for the NA-independent replication of an H5 influenza virus.

EscEgg50A and EscEgg50Arec exhibit significantly lower neutralization titers. Viral escape due to the neutralizing activity of the polyclonal serum used was expected as a consequence of the imposed immune pressure. Therefore, the neutralization of the different viruses was quantified by a standard virus neutralization assay. Ancestor virus, CoEgg50, EscEgg50A, and a recombinant virus with the ancestor backbone and the EscEgg50A NA protein (EscEgg50Arec) were tested and the results were compared. In addition, neutralization tests were carried out with a second polyclonal serum sample (serum sample B) from an H5-vaccinated and H5N1-challenged chicken. All tested viruses were neutralized by the passage serum (serum sample A) and the second individual serum sample (serum sample B); however, significantly lower neutralization titers were observed for the group of neuraminidase-negative viruses (Fig. 6). Interestingly, when one-way ANOVA was applied, the individual neutralizing titers of the sera against the different viruses did not differ significantly (Fig. 6). These results indicate a more efficient neutralization capacity of all immune sera against viruses encoding a neuraminidase protein.

Generation of additional H5N1 segment 6 variants (EscEgg50B and EscEgg50C). To evaluate whether the induction of large deletions and rearrangements of the neuraminidase-coding segment is repeatable, we passaged the virus from the 30th passage of R65/06, which was one of the latest passages from which virus tested positive for the parental NA and negative for the rearranged NA (Table 3), under the selection pressure of two different chicken serum samples (serum samples B and C) and obtained two additional escape variant viruses, EscEgg50B and EscEgg50C, respectively. Partial deletion and intrasegmental as well as intersegmental recombination of segment 6 sequences were detected in the two additional variants (Fig. 1B and C). EscEgg50B exhibited one large deletion of 950 bp within the NA gene (GISAID accession no. EPI383000), while both termini of the segment remained nearly identical to the ancestor sequence (Fig. 1; Table 2). The open reading frame encoded the first 55 aa of the ancestor neuraminidase, followed by 3 heterologous amino acids and a stop codon.

The segment 6 sequence of virus EscEgg50C started with 118 nt identical to the ancestor sequence, followed by a short sequence (30 nt) with 100% homology to a sequence from segment 4 of the ancestor virus and then sequences from the 3′ terminus of segment 6 again identical to the sequence of the ancestor virus (Fig. 1; Table 2; GISAID accession no. EPI383001). The amino acid sequence encoded by EscEgg50C shared 100% homology to the an-

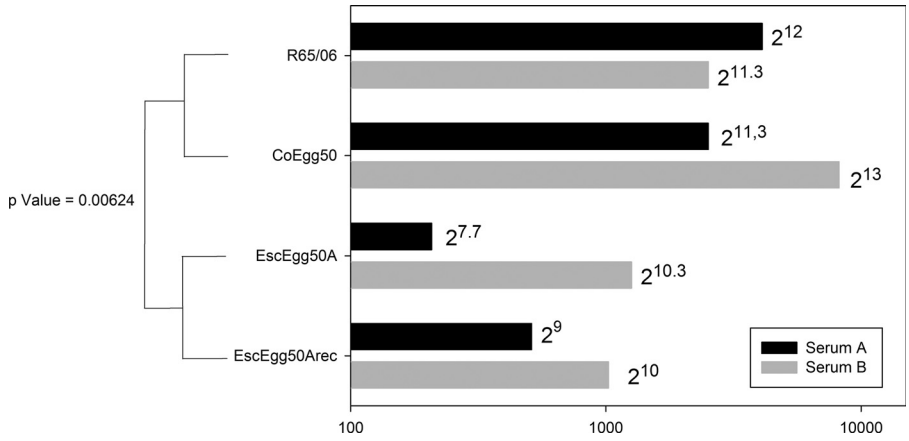

FIG 6 Neutralizing activity of the serum sample used to generate EscEgg50A serum (serum sample A) and a second serum sample (serum sample B) determined by a standard virus neutralization assay. The numerical values of the neutralizing activity on the scale at the bottom are also displayed as $\log_2$ values to the right of the bars. Results were statistically evaluated by one-way ANOVA. The *P* value represents the significance of the serum neutralization of viruses lacking NA activity (EscEgg50A, EscEgg50Arec) in comparison to that of viruses possessing NA activity (R65/06, CoEgg50).

cestor sequence for the first 32 aa, followed by a further 17 heterologous amino acids.

Overall, independent truncation and segment shuffling events repeatedly occurred in segment 6 during parallel passaging of an H5N1 HPAIV under multiple immunogenic pressures.

The EscEgg50A variant is established after egg passage 36. In order to determine more precisely the passage in which the segment 6 rearrangement took place, we designed an RT-qPCR assay based specifically on the EscEgg50A segment 6 sequences. With this specific RT-qPCR assay, EscEgg50A virus, the ancestor virus R65/06, and viruses isolated after intermediate passages were analyzed. As a control, every RNA preparation was tested with H5- and N1-specific duplex RT-qPCR assays in parallel (Table 3). Assessment of the quantification cycle values demonstrated that sequences specific for the finally deleted and rearranged NA of the EscEgg50A variant first occurred after passage 36 (Table 3). Interestingly, mixed populations of the original and the rearranged sequences were not detected by this sensitive assay (Table 3), suggesting minute frequencies of viral variants. To shed light on the sequence distribution over subsequent passages, segment 6 of the ancestor virus, EscEgg50A, and the viruses isolated after intermediate passages were amplified with segment 6-specific primers and subjected to next-generation sequencing using the GS FLX instrument. Remarkably, the ancestor population already contained a certain small portion of shortened sequences besides the full-length master sequence (1,396 nucleotides), and every successive population retained these minor fractions. The master sequences determined from the 30th passage comprised 1,235 nucleotides caused by two short deletions. Within the sequences detected from the 35th and 36th passages, the insertion of the segment 3-derived sequences, i.e., the occurrence of sequence shuffling, could be found in trace amounts, while the master sequences still resembled the sequence from the 30th passage. From passage 37, the master sequence was identical to the segment 6 sequence of EscEgg50A, being 678 nucleotides long with segment 3 fragments inserted. We therefore concluded that EscEgg50A is a replication-

TABLE 3 Results of detection of H5 and native and rearranged N1 sequences by RT-qPCR and sequence length of the master sequence estimated by GS FLX sequencing[a]

Variant	RT-qPCR C_q value			No. of nucleotides in master sequence	PA insertion
	H5[b]	N1[b]	EscEgg50A NA specific[c]		
Ancestor virus (R65/06)	13.0	15.4	Neg	1,396	Neg
CoEgg50	13.6	12.2	Neg	ND	ND
EscEgg30	17.6	20.7	Neg	1,235	Neg
EscEgg35	19.4	22.5	Neg	1,235	Pos
EscEgg36	22.2	25.2	Neg	1,235	Pos
EscEgg37	19.1	Neg	21.3	678	Pos
EscEgg50	18.5	Neg	20.7	678	Pos

[a] C_q, quantification cycle; Neg, negative; Pos, positive; ND, not determined.
[b] Viral RNA detected by duplex RT-qPCR. Quantification cycle values of >35 were scored as negative.
[c] Viral RNA detected by EscEgg50A NA-specific RT-qPCR, as described in the Materials and Methods section. Quantification cycle values of >35 were scored as negative.

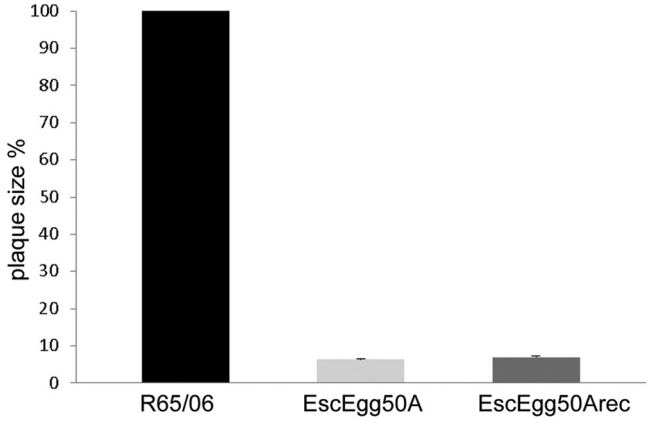

FIG 7 Plaque sizes of R65/06, EscEgg50A, and EscEgg50Arec. MDCK cells in six-well plates were infected at an MOI of 0.01 and overlaid with agarose for 24 h. The plaque diameters of 50 randomly selected plaques were determined. The average diameter of plaques formed by the wild-type strain R65/06 was set to 100%. Error bars indicate SEMs.

competent virus without neuraminidase activity, having become predominant in the viral population after passage 36.

***In vitro* characterization of the variants with NA deletions displaying an altered growth phenotype.** In order to examine the effect of the NA deletion on viral cell-to-cell spread (ctcs), diameters of 50 plaques for the ancestor virus, the escape variant EscEgg50A, and the recombinant EscEgg50Arec were measured, and mean diameters and standard deviations were calculated. Values for the parental HPAIV H5N1 strain R65/06 were set to 100%, and the plaque diameters observed for the mutant viruses were expressed relative to this value (Fig. 7). Deletion of a functional NA protein resulted in a 93% reduction in plaque diameter (Fig. 7).

Furthermore, hemagglutination using standard protocols could not be demonstrated for any of the NA-negative mutant viruses, in contrast to the wild type (data not shown).

To evaluate the growth characteristics of the EscEgg50A mutant further, viral replication in cell culture was analyzed. EscEgg50A replicated in cell culture in the absence of exogenous sialidase and showed a characteristic growth phenotype of prominent 3-dimensional cloggy structures representing aggregates of infected cells (Fig. 2). In contrast, the parental strain R65/06 induced typical influenza virus plaque formation in the cell monolayer. Growth kinetics were determined for ancestor virus R65/06, escape variant EscEgg50A, and reconstituted recombinant virus EscEgg50Arec in one-step and multistep assays. After inoculation using an MOI of 1, a growth delay of the EscEgg50A and EscEgg50Arec variants until 48 h postinfection was observed for the cell culture supernatant, i.e., released virus, and viral titers at 72 h postinfection exhibited similar values as wild-type HPAIV H5N1 (MOI, 1; Fig. 5A). Multistep kinetics revealed a much more prominent delay of viral growth, especially for EscEgg50Arec (Fig. 5C). Since EscEgg50Arec, which consists of the rearranged NA in the background of parental virus R65/06, seems to have an impaired spread, we speculate that additional amino acid substitutions in EscEgg50A which are not present in EscEgg50Arec may contribute to virus spread (Table 1). Viral titers from cell lysates showed no marked replication differences (MOI, 1; Fig. 5B) or were reduced by 10-fold (MOI, 0.01; EscEgg50A) or 400-fold (MOI, 0.01; EscEgg50Arec) (Fig. 5D). Taken together, our data demonstrate a clear effect of the MOI used, which is consistent with the observed markedly reduced cell-to-cell spread ability of the variants with NA deletions. In addition, virus release might be delayed but is not markedly influenced by the NA deletion at high MOIs.

In order to examine the effect of exogenous sialidase on the growth characteristics, we cultured the ancestor virus R65/06, the escape variant EscEgg50A, and the recombinant virus EscEgg50Arec in the presence of *Clostridium perfringens* neuraminidase. Interestingly, supplementation with that bacterial sialidase resulted in significantly improved viral titers for the NA-negative variants as well as for the ancestor virus (Fig. 8A). However, that titer increase was more pronounced for the NA-negative viruses tested: 1,613-fold for EscEgg50A and 879-fold for recombinant virus EscEgg50Arec (Fig. 8B). Therefore, the bacterial sialidase used was able to compensate for the growth deficiency of the NA-negative viruses.

The NA-negative variants are fully attenuated. To assess changes in the virulence of EscEgg50A, EscEgg50B, EscEgg50C, or CoEgg50, we determined the intravenous pathogenicity index (IVPI) in chicken (22). With an IVPI of 2.97, the ancestor virus was classified as highly pathogenic (28), meaning that every animal succumbed to the disease within 3 days. The control virus CoEgg50 was also demonstrated to be an HPAIV with a very close IVPI of 2.55. In contrast, the IVPIs of all three NA-negative variants, EscEgg50A, EscEgg50B, and EscEgg50C, were 0; i.e., none of the chickens became sick. Therefore, despite the presence of the unchanged polybasic HA cleavage site, which is the major molecular marker of HPAIV, the EscEgg50A, EscEgg50B, and EscEgg50C mutants were unequivocally classified as low pathogenic. Almost all chicken serum samples (samples from 27 out of 30 animals) scored positive by the NP and H5 antibody ELISAs, indicating occult infection, whereas all those individual serum samples tested negative for N1-specific antibodies.

To simulate natural infection via the respiratory tract, groups of 10 chickens were infected oronasally with the three different EscEgg50 viruses. None of those birds showed any clinical symptoms. In contrast, all CoEgg50-infected animals succumbed to death within 6 days. Four out of 10 EscEgg50A-inoculated chickens had a positive antibody reaction in an NP-specific ELISA, and 3 of them were also positive in an H5-specific antibody ELISA (Table 4). Five chickens inoculated with EscEgg50B seroconverted against NP, and 4 of them also reacted against H5. From the group of chickens inoculated with the EscEgg50C variant, only two seroconverted against NP and one of these also scored positive by the H5 ELISA (Table 4). Fourteen days after inoculation of EscEgg50 mutants, all chickens were oronasally challenged with a lethal dose of ancestor virus HPAIV R65/06. Remarkably, all chickens scoring positive for H5 antibodies survived the challenge infection asymptomatically, while all other animals died (Table 4).

These results suggest a reduced infection efficacy of the EscEgg50 mutants via the oronasal route. Overall, the EscEgg50 viruses exhibited an apathogenic phenotype in chickens of 6 weeks of age.

DISCUSSION

Continuous circulation of HPAIV H5N1 in poultry and wild birds with repeated spillover to humans is reported from Southeast Asian countries and Egypt, even though extensive vaccination campaigns or eradication programs are in place. Antigenic drift variants have arisen in immunized, not fully protected animals and hamper vaccine-based eradication strategies. In this study, we aimed to model influenza A virus immunoescape closer to the *in vivo* situation by egg passaging an H5N1 HPAIV strain under the more authentic multifarious selection pressure of a polyclonal serum from individual chickens, since cell culture systems may not simulate the real situation for vaccinated flocks with thousands of birds. However, to assess the enormous genetic plasticity of influenza viruses, we intended to implement an antigenic drift model based on repeated passaging in the presence of polyclonal immune sera in an embryonated egg culture. During egg passages, immunogenic pressure results in the emergence of progeny viruses with mutations with an altered antigenic pattern (29). *In vitro* selection in the presence of monoclonal antibodies and polyclonal (rabbit- or mouse-derived) antisera was utilized to identify several antigenic epitopes of the hemagglutinin (8, 30–33) and the neuraminidase (34). Using polyclonal chicken sera in a cell culture system (10), we recently obtained escape variants whose variations reflect immunoescape beyond the major antigenic HA epitopes affecting several viral proteins. Surprisingly, our repeated long-term pas-

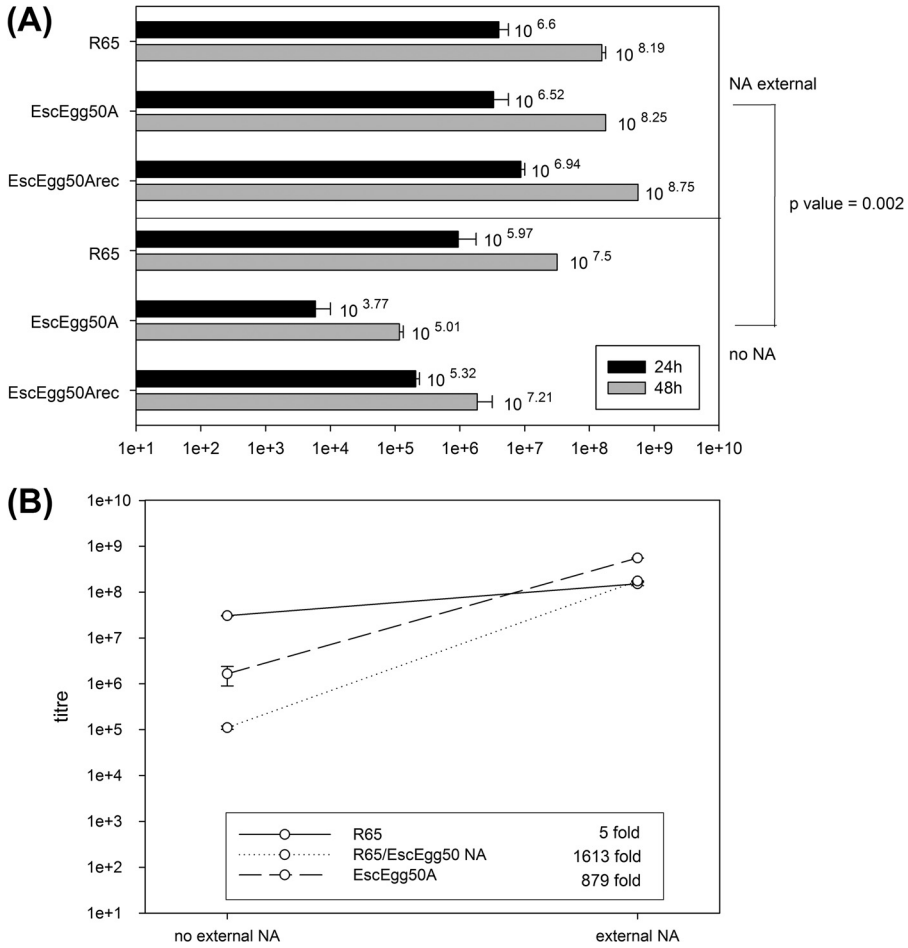

FIG 8 Viral titers after supplementation of external bacterial sialidase. (A) MDCK cells incubated with and without bacterial neuraminidase were infected with R65/06, EscEgg50A, and EscEgg50Arec at an MOI of 0.1. Cell culture supernatant was collected after 24 h and 48 h of incubation, and viral titers (indicated to the right of the bars and by the scale at the bottom) were determined by titration. (B) Increase in viral titers effected by the bacterial neuraminidase. The results are mean values of two independent experiments. Graphs and statistical analyses were performed using SigmaPlot software (Windows, v 11.0; Build, v 11.2.0.5; Systat Software Inc.). The *P* values of the different titers from virus cultured with or without external neuraminidase were determined using analysis of variance by the Kruskal-Wallis method.

saging experiments resulted in virus variants with NA deletions derived from HPAIV H5N1 that have never described before.

Although the sera used for selection showed HI titers similar to those in sera from field studies or similar experiments (6, 12), there were no relevant changes in the HA sequence. Despite the dogma that antibodies against the neuraminidase are nonneutralizing and of lower relevance (35), the changes observed within segment 6 were significant and a functional NA protein was no longer expressed. On the other hand, the neutralization data suggest that the changes observed in segment 6 had some effect on the neutralizing capacity of the tested polyclonal antisera. Repeated passaging resulted in similar but different NA variants. One possible explanation for the emergence of virus variants with NA deletions during passaging is that the deletions have an indirect effect on the neuraminidase gene, since neutralization might be circumvented by limiting the release of free-floating virions and viruses instead spread via the cell-to-cell route (36).

Furthermore, since the EscEgg50A virus evolved only one amino acid substitution within the HA sequence after 50 egg passages, stabilization of the HA sequence and some kind of immunoevasion due to the loss of the corresponding neuraminidase protein must be taken into account. The minor role of the HA variation is further proven by the not markedly different neutralizing data for the EscEgg50Arec virus, which had the same HA segment as the ancestor virus. Furthermore, antiserum samples A, B, and C were each able to efficiently select the three different neuraminidase-negative virus variants, EscEgg50A, -B, and -C. It could be also demonstrated by a neuraminidase assay that direct inhibition of the neuraminidase function by antibody-positive sera is possible. However, the N1-specific antibody ELISA scored

TABLE 4 Serology after oronasal application of EscEgg50A, -B, or -C[a]

Variant	No. of chickens ELISA positive/total no. tested			No. of survivors/total no. challenged
	NP	H5	N1	
EscEgg50A	4/10	3/10	0/10	3/10
EscEgg50B	5/10	4/10	0/10	6/10
EscEgg50C	2/10	1/10	0/10	1/10

[a] Fourteen days after inoculation and before challenge virus infection.

negative for the serum samples tested (serum samples B and C), thus indicating the presence of large amounts of N1-specific antibodies to be unlikely, but this result does not exclude the possibility of the presence of neuraminidase antibodies at lower titers because the ELISA used was directed against a single epitope only. Therefore, the exact selection mechanism remains unclear, but the role of possible factors like neuraminidase-specific antibodies will be further investigated in future studies.

The variations in the H5 virus variants that we detected after 50 egg passages under pressure with an antiserum were unique, and the variations were focused on segment 6. Extensive deletions and rearrangements were ascertained to exist exclusively in segment 6. Interestingly, the new segments 6 resulted from complex sequence shuffling, and two out of three variants had insertions of a very short sequence fragment originating from other segments. Therefore, recombination events are an underestimated mechanism of sequence variation in influenza viruses (37). Characterization of the novel variants confirmed the complete loss of the neuraminidase protein and of any neuraminidase activity. Recombinant viruses with a deleted ATG sequence or the insertion of EGFP proved that even the N-terminal residual peptide encoded by the truncated segment 6 of the EscEgg50A virus is not essential for virus replication.

In previous reports, deletion of major parts of segment 6 was seen only after virus passaging in the presence of an exogenous sialidase (38–40) or after passaging of H3N2 viruses on MDCK cells (39). However, in the latter case and in contrast to our variants with NA deletions, attempts to isolate a neuraminidase-negative virus strain were not successful, and the authors therefore suggested that full-length segment 6 remained in the virus population at a lower frequency. A complete loss of a neuraminidase-encoding segment or neuraminidase function is deemed impossible (41), with the only example of this being in a human H3 isolate which was claimed to lack the complete segment 6 and addressed as a seven-segmented influenza A virus (42). Alternatively, the growth of a neuraminidase-negative influenza A virus without supplementation of exogenous sialidase was demonstrated *in vitro* and in mice only if the loss of neuraminidase activity was accompanied by mutations around the HA receptor-binding pocket, which lowered the avidity for receptors (43, 44). Since a recombinant virus composed of 7 ancestor virus R65/06-specific segments in combination with segment 6 of the escape variant EscEgg50A was generated by reverse genetics, the role of compensatory mutations was negligible here. Replication competence could be demonstrated, and the HA sequences did not indicate the presence of any compensatory mutations. Therefore, replication competence, despite a truncated segment 6, is achievable without any additional compensatory changes in the HA protein in the presence of a polybasic cleavage site. Thus, the NA of HPAIV H5N1 may not be essential for virus replication and assembly but is necessary for efficient cell-to-cell spread and growth at low MOIs.

The neuraminidase deletion apparently provided an advantage, as the viral population shifted to neuraminidase-truncated viruses on embryonated eggs, as was demonstrated by the predominance of EscEgg50A-specific sequences in the viral population from passage 37 on (Table 3). This is in accordance with the intersegmental recombination events found within segment 6 after passaging of a segment 6 stalk deletion mutant in egg culture, where recombination events occurred within one passage step (37). Sequence variation of segment 6 could be verified as early as passage 30 and led to a truncated form of segment 6. In addition, rigorously shortened versions of segment 6 could be detected in every passage. Under von Magnus conditions, influenza viruses are known to produce defective interfering (DI) particles carrying mostly a large deletion in the polymerase genes (45), and these are maintained in the population by coreplication. However, it is a novel finding that such a DI segment can entirely replace the full-length counterpart, as demonstrated by reconstitution of recombinant variant EscEgg50Arec. Mechanistically, such sequence rearrangements were postulated to be due to viral polymerase jumping across the ends of hairpin RNA structures (41), which to some extent might be the underlying reason for the truncation, shuffling, and recombination events observed within segment 6.

Like previously described NA variants (39), EscEgg50A segment 6 retained the sequences encoding the cytoplasmic tail together with the transmembrane region of the NA protein and, in addition, the noncoding sequences at both the 3′ and the 5′ segment ends required for efficient incorporation of the viral genome into budding particles (46).

One possible source of a sialidase enzyme in our experiment could have been the embryonated egg itself (47). However, the control virus, also passaged 50 times in egg culture, showed no variation in the NA sequence, implying that negative selection by the polyclonal serum was necessary to force the NA deletion. Furthermore, EscEgg50A, -B, and -C viruses replicated in cell cultures without any exogenous neuraminidases, refuting a determining role of the egg neuraminidases for generation of the mutants with truncated NA.

Growth analysis of the viruses with NA deletions demonstrated a delay of replication, but the viral titers achieved 72 h after infection at an MOI of 1 were clearly in the range of those of the ancestor virus, about 10^8 $TCID_{50}$s/ml. Despite the high viral titers within the supernatant, the NA-negative viruses displayed *in vitro* two marked differences in comparison to the ancestor virus: they exhibited no hemagglutinating activity at all, and viral cell-to-cell spread (ctcs) was drastically reduced. NA-negative viruses are known to lose hemagglutinating activity (37, 48), and the lack of desialylation of the HA protein (49) likely explains this phenomenon. In addition, the functions of HA and NA must be orchestrated (37), and therefore, NA-negative viruses may reduce HA binding affinity. Whether the impairment of ctcs is due to impaired HA-NA cooperation remains an open question. An effect of NA on plaque sizes was observed earlier (50). The altered plaque phenotypes, where plaques were very small, in cultures exhibiting cloggy-like structures of tightly agglomerated cells were likely a result of the impaired ctcs.

Despite the multibasic cleavage site within the HA protein, which is typical for highly pathogenic viruses, EscEgg50A exhibited an apathogenic phenotype *in vivo*. The deletion of the neuraminidase obviously led to complete attenuation of the virus in 6-week-old chickens; furthermore, oronasal infection was not successful in the majority of the inoculated animals.

As one proposed function of the neuraminidase protein in the airways is cleavage of complex substrates (mucins) to mediate access to target cells and virus release from infected cells (51), the markedly reduced infectiousness of the EscEgg50 variants in the oronasal infection model seems reasonable. Furthermore, the drastically reduced ctcs corresponds to the *in vivo* phenotype of the viruses with NA deletions. We therefore conclude that the neuraminidase protein is not essential for the *in vitro* growth of

HPAIV H5N1-derived viruses but is required for host entry and probably spread within the host and is therefore a substantial virulence factor *in vivo*. Overall, such attenuated H5N1 viruses carrying large deletions in their NA gene segments may serve as unique tools to study the role of the neuraminidase in virus assembly, growth, and pathogenesis.

Furthermore, the deletion of the neuraminidase provides a new approach to attenuated live vaccines. Provided that wild-type virus infections cause a serological response to the NA detectable in vaccinated animals, an NA-deficient virus would be a perfect vaccine candidate, enabling the differentiation of infected from vaccinated animals (DIVA). The present limitation of such a vaccine strain would still be the highly pathogenic genotype of the HA segment, which is essential for the efficient growth of H5 viruses with NA deletions. This HA gene might be delivered to circulating low-pathogenic viruses, generating a novel highly pathogenic strain. Therefore, the essential requirement of a polybasic HA cleavage site for efficient growth of H5 viruses with NA deletions remains to be studied further. A future milestone would be the construction of highly productive H5 or H7 strains with NA deletions and without a polybasic HA cleavage site for use as attenuated live vaccines with a high degree of safety.

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REFERENCES

1. **Peiris JSM, de Jong MD, Guan Y.** 2007. Avian influenza virus (H5N1): a threat to human health. Clin. Microbiol. Rev. **20:**243–267.
2. **Swayne DE.** 2009. Avian influenza vaccines and therapies for poultry. Comp. Immunol. Microbiol. Infect. Dis. **32:**351–363.
3. **Earn DJD, Dushoff J, Levin SA.** 2002. Ecology and evolution of the flu. Trends Ecol. Evol. **17:**334–340.
4. **Kilany WH, Arafa A, Erfan AM, Ahmed MS, Nawar AA, Selim AA, Khoulosy SG, Hassan MK, Aly MM, Hafez HM, Abdelwhab EM.** 2010. Isolation of highly pathogenic avian influenza H5N1 from table eggs after vaccinal break in commercial layer flock. Avian Dis. **54:**1115–1119.
5. **Abdelwhab el-SM, Erfan AM, Grund C, Ziller M, Arafa AS, Beer M, Aly MM, Hafez HM, Harder TC.** 2010. Simultaneous detection and differentiation by multiplex real-time RT-PCR of highly pathogenic avian influenza subtype H5N1 classic (clade 2.2.1 proper) and escape mutant (clade 2.2.1 variant) lineages in Egypt. Virol. J. **7:**260.
6. **Grund C, Abdelwhab el-SM, Arafa AS, Ziller M, Hassan MK, Aly MM, Hafez HM, Harder TC, Beer M.** 2011. Highly pathogenic avian influenza virus H5N1 from Egypt escapes vaccine-induced immunity but confers clinical protection against a heterologous clade 2.2.1 Egyptian isolate. Vaccine **29:**5567–5573.
7. **Ferreira HL, Lambrecht B, van Borm S, Torrieri-Dramard L, Klatzmann D, Bellier B, van den Berg T.** 2010. Identification of a dominant epitope in the hemagglutinin of an Asian highly pathogenic avian influenza H5N1 clade 1 virus by selection of escape mutants. Avian Dis. **54:**565–571.
8. **Rudneva IA, Kushch AA, Masalova OV, Timofeeva TA, Klimova RR, Shilov AA, Ignatieva AV, Krylov PS, Kaverin NV.** 2010. Antigenic epitopes in the hemagglutinin of Qinghai-type influenza H5N1 virus. Viral Immunol. **23:**181–187.
9. **Kryazhimskiy S, Dushoff J, Bazykin GA, Plotkin JB.** 2011. Prevalence of epistasis in the evolution of influenza A surface proteins. PLoS Genet. **7:**e1001301. doi:10.1371/journal.pgen.1001301.
10. **Höper D, Kalthoff D, Hoffmann B, Beer M.** 2012. Highly pathogenic avian influenza virus subtype H5N1 escaping neutralization: more than HA variation. J. Virol. **86:**1394–1404.
11. **Weber S, Harder T, Starick E, Beer M, Werner O, Hoffmann B, Mettenleiter TC, Mundt E.** 2007. Molecular analysis of highly pathogenic avian influenza virus of subtype H5N1 isolated from wild birds and mammals in northern Germany. J. Gen. Virol. **88:**554–558.
12. **Rudolf M, Pöppel M, Fröhlich A, Breithaupt A, Teifke J, Blohm U, Mettenleiter T, Beer M, Harder T.** 2010. Longitudinal 2 years field study of conventional vaccination against highly pathogenic avian influenza H5N1 in layer hens. Vaccine **28:**6832–6840.
13. **Höper D, Hoffmann B, Beer M.** 2009. Simple, sensitive, and swift sequencing of complete H5N1 avian influenza virus genomes. J. Clin. Microbiol. **47:**674–679.
14. **Leifer I, Hoffmann B, Höper D, Bruun Rasmussen T, Blome S, Strebelow G, Höreth-Böntgen D, Staubach C, Beer M.** 2010. Molecular epidemiology of current classical swine fever virus isolates of wild boar in Germany. J. Gen. Virol. **91:**2687–2697.
15. **Sanger F, Nicklen S, Coulson AR.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. **74:**5463–5467.
16. **Darling ACE, Mau B, Blattner FR, Perna NT.** 2004. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res. **14:**1394–1403.
17. **R Development Core Team.** 2009. R: a language and environment for statistical computing, version 2.10.1. R Foundation for Statistical Computing, Vienna, Austria
18. **Stech J, Stech O, Herwig A, Altmeppen H, Hundt J, Gohrbandt S, Kreibich A, Weber S, Klenk HD, Mettenleiter TC.** 2008. Rapid and reliable universal cloning of influenza A virus genes by target-primed plasmid amplification. Nucleic Acids Res. **36:**e139. doi:10.1093/nar/gkn646.
19. **Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG.** 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. U. S. A. **97:**6108–6113.
20. **Bahnemann HG.** 1990. Inactivation of viral antigens for vaccine preparation with particular reference to the application of binary ethylenimine. Vaccine **8:**299–303.
21. **Rowe T, Abernathy RA, Hu-Primmer J, Thompson WW, Lu X, Lim W, Fukuda K, Cox NJ, Katz JM.** 1999. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. J. Clin. Microbiol. **37:**937–943.
22. **International Office of Epizootics.** 2008. Manual of diagnostic tests and vaccines for terrestrial animals, 6th ed. International Office of Epizootics, Paris, France.
23. **Pavlova SP, Veits J, Keil GM, Mettenleiter TC, Fuchs W.** 2009. Protection of chickens against H5N1 highly pathogenic avian influenza virus infection by live vaccination with infectious laryngotracheitis virus recombinants expressing H5 hemagglutinin and N1 neuraminidase. Vaccine **27:**773–785.
24. **Hoffmann B, Harder T, Starick E, Depner K, Werner O, Beer M.** 2007. Rapid and highly sensitive pathotyping of avian influenza A H5N1 virus by using real-time reverse transcription-PCR. J. Clin. Microbiol. **45:**600–603.
25. **Hoffmann B, Depner K, Schirrmeier H, Beer M.** 2006. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J. Virol. Methods **136:**200–209.
26. **Hoffmann B, Harder T, Lange E, Kalthoff D, Reimann I, Grund C, Oehme R, Vahlenkamp TW, Beer M.** 2010. New real-time reverse transcriptase polymerase chain reactions facilitate detection and differentiation of novel A/H1N1 influenza virus in porcine and human samples. Berl. Munch. Tierarztl. Wochenschr. **123:**286–292.
27. **Gohrbandt S, Veits J, Hundt J, Bogs J, Breithaupt A, Teifke JP, Weber S, Mettenleiter TC, Stech J.** 2011. Amino acids adjacent to the haemagglutinin cleavage site are relevant for virulence of avian influenza viruses of subtype H5. J. Gen. Virol. **92:**51–59.
28. **Rudolf M, Pöppel M, Fröhlich A, Mettenleiter T, Beer M, Harder T.** 2009. Efficacy of a commercial inactivated H5 influenza vaccine against highly pathogenic avian influenza H5N1 in waterfowl evaluated under field conditions. Rev. Sci. Tech. **28:**275–291.

29. **Archetti I, Horsfall JFL.** 1950. Persistent antigenic variation of influenza A viruses after incomplete neutralization in ovo with heterologous immune serum. J. Exp. Med. **92:**441–462.
30. **Kaverin NV, Rudneva IA, Govorkova EA, Timofeeva TA, Shilov AA, Kochergin-Nikitsky KS, Krylov PS, Webster RG.** 2007. Epitope mapping of the hemagglutinin molecule of a highly pathogenic H5N1 influenza virus by using monoclonal antibodies. J. Virol. **81:**12911–12917.
31. **Philpott M, Hioe C, Sheerar M, Hinshaw VS.** 1990. Hemagglutinin mutations related to attenuation and altered cell tropism of a virulent avian influenza A virus. J. Virol. **64:**2941–2947.
32. **Lambkin R, McLain L, Jones SE, Aldridge SL, Dimmock NJ.** 1994. Neutralization escape mutants of type A influenza virus are readily selected by antisera from mice immunized with whole virus: a possible mechanism for antigenic drift. J. Gen. Virol. **75:**3493–3502.
33. **Cleveland SM, Taylor HP, Dimmock NJ.** 1997. Selection of neutralizing antibody escape mutants with type A influenza virus HA-specific polyclonal antisera: possible significance for antigenic drift. Epidemiol. Infect. **118:**149–154.
34. **Laver WG, Air GM, Webster RG, Markoff LJ.** 1982. Amino acid sequence changes in antigenic variants of type A influenza virus N2 neuraminidase. Virology **122:**450–460.
35. **Spackman E.** 2008. A brief introduction to the avian influenza virus. Methods Mol. Biol. **436:**1–6.
36. **Mori K, Haruyama T, Nagata K.** 2011. Tamiflu-resistant but HA-mediated cell-to-cell transmission through apical membranes of cell-associated influenza viruses. PLoS One **6:**e28178. doi:10.1371/journal.pone.0028178.
37. **Mitnaul LJ, Matrosovich MN, Castrucci MR, Tuzikov AB, Bovin NV, Kobasa D, Kawaoka Y.** 2000. Balanced hemagglutinin and neuraminidase activities are critical for efficient replication of influenza A virus. J. Virol. **74:**6015–6020.
38. **Liu C, Air GM.** 1993. Selection and characterization of a neuraminidase-minus mutant of influenza virus and its rescue by cloned neuraminidase genes. Virology **194:**403–407.
39. **Yang P, Bansal A, Liu C, Air GM.** 1997. Hemagglutinin specificity and neuraminidase coding capacity of neuraminidase-deficient influenza viruses. Virology **229:**155–165.
40. **LeBouder F, Lina B, Rimmelzwaan GF, Riteau B.** 2010. Plasminogen promotes influenza A virus replication through an annexin 2-dependent pathway in the absence of neuraminidase. J. Gen. Virol. **91:**2753–2761.
41. **Gulati S, Smith DF, Air GM.** 2009. Deletions of neuraminidase and resistance to oseltamivir may be a consequence of restricted receptor specificity in recent H3N2 influenza viruses. Virol. J. **6:**22.
42. **Moules V, Ferraris O, Terrier O, Giudice E, Yver M, Rolland JP, Bouscambert-Duchamp M, Bergeron C, Ottmann M, Fournier E, Traversier A, Boule C, Rivoire A, Lin Y, Hay A, Valette M, Marquet R, Rosa-Calatrava M, Naffakh N, Schoehn G, Thomas D, Lina B.** 2010. In vitro characterization of naturally occurring influenza H3NA-viruses lacking the NA gene segment: toward a new mechanism of viral resistance? Virology **404:**215–224.
43. **Hughes MT, Matrosovich M, Rodgers ME, McGregor M, Kawaoka Y.** 2000. Influenza A viruses lacking sialidase activity can undergo multiple cycles of replication in cell culture, eggs, or mice. J. Virol. **74:**5206–5212.
44. **Gubareva LV, Nedyalkova MS, Novikov DV, Murti KG, Hoffmann E, Hayden FG.** 2002. A release-competent influenza A virus mutant lacking the coding capacity for the neuraminidase active site. J. Gen. Virol. **83:**2683–2692.
45. **Von Magnus P.** 1951. Propagation of the PR8 strain of influenza A virus in chick embryos. III. Properties of the incomplete virus produced in serial passages of undiluted virus. Acta Pathol. Microbiol. Scand. **29:**157–181.
46. **Fujii Y, Goto H, Watanabe T, Yoshida T, Kawaoka Y.** 2003. Selective incorporation of influenza virus RNA segments into virions. Proc. Natl. Acad. Sci. U. S. A. **100:**2002–2007.
47. **Ada GL, Lind PE.** 1961. Neuraminidase in the chorioallantois of the chick embryo. Nature **190:**1169–1171.
48. **Tisoncik JR, Guo Y, Cordero KS, Yu J, Wang J, Cao Y, Rong L.** 2011. Identification of critical residues of influenza neuraminidase in viral particle release. Virol. J. **8:**14.
49. **Su B, Wurtzer S, Rameix-Welti MA, Dwyer D, van der Werf S, Naffakh N, Clavel F, Labrosse B.** 2009. Enhancement of the influenza A hemagglutinin (HA)-mediated cell-cell fusion and virus entry by the viral neuraminidase (NA). PLoS One **4:**e8495. doi:10.1371/journal.pone.0008495.
50. **Markoff LJ, Murphy BR, Kendal AJ, Chanock RM.** 1979. Probable association of plaque size with neuraminidase subtype among H3N2 influenza A viruses. Brief report. Arch. Virol. **62:**277–280.
51. **Schauer R.** 2004. Victor Ginsburg's influence on my research of the role of sialic acids in biological recognition. Arch. Biochem. Biophys. **426:**132–141.