Peer-reviewed version available at *Int. J. Mol. Sci.* **2020**, *21*, 2353; doi:10.3390/ijms21072353

Running Title: Genetic Determinants for HPAIV non-H5/H7

Insertion of basic amino acids in the hemagglutinin cleavage site of H4N2 avian influenza virus (AIV) reduced virus fitness in chickens which is restored by reassortment with highly pathogenic H5N1 AIV

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Abstract

Highly pathogenic (HP) avian influenza viruses (AIVs) are naturally restricted to H5 and H7 subtypes with a polybasic cleavage site (CS) in the hemagglutinin (HA) and any AIV with an intravenous pathogenicity index (IVPI) ≥1.2. Only few non-H5/H7 viruses fulfill the criteria of HPAIVs; nevertheless, it remains unknown why these viruses did not spread in domestic birds. In 2012, a unique H4N2 virus with a polybasic CS ^{322}PE**KRR**T**R**/G^{329} was isolated from quails in California which, however, was avirulent in chickens. This is the only known non-H5/H7 virus with four basic amino acids in the HACS. Here, we investigated the virulence of this virus in chickens after expansion of the polybasic CS by substitution of T^{327}R (^{322}PE**KRRRR**/G^{329}) or T^{327}K (^{322}PE**KRRKR**/G^{329}) with or without reassortment with HPAIVs H5N1 and H7N7. The impact of single mutations or reassortment on virus fitness *in vitro* and *in vivo* was studied. Efficient cell culture replication of T^{327}R/K carrying H4N2 viruses increased by trypsin, particularly in MDCK cells, and reassortment with HPAIV H5N1. Likewise, replication, virus excretion and bird-to-bird transmission of H4N2 was remarkably compromised by the CS mutations, but restored after reassortment with HPAIV H5N1, although not with HPAIV H7N7. Viruses carrying the H4-HA with or without R^{327} or K^{327} mutations and the other gene segments from HPAIV H5N1 exhibited high virulence and efficient transmission in chickens. Together, increasing the number of basic amino acids in the H4N2 HACS was detrimental for viral fitness particularly *in vivo* but compensated by reassortment with HPAIV H5N1. This may explain the absence of non-H5/H7 HPAIVs in poultry.

Keywords

Highly Pathogenic Avian Influenza Virus, Low Pathogenic Avian Influenza Virus, Evolution, Virulence Determinants, non-H5/H7, Cleavage Site, Chicken-to-Chicken Transmission, Virulence, Protease

Introduction

Influenza A viruses are members of the family *Orthomyxoviridae* and divided into equine, classical swine / human, gull, bat and avian influenza virus (AIV) lineages [1,2]. The genome of AIVs consists of eight segments coding for at least nine structural and one non-structural (NS) viral proteins [1]. Based on the antigenic properties of the surface glycoproteins, AIVs are currently classified into 16 hemagglutinin (HA) (H1 – H16) and 9 neuraminidase (NA) (N1 – N9) subtypes which have been isolated from aquatic and domestic birds in different HxNy combinations [3]. AIVs are further classified according to virulence in chickens into low pathogenic (LP) and highly pathogenic (HP) forms. LPAIVs cause mild or no clinical signs, while HPAIVs cause severe illness with mortality rates up to 100% within few days [4]. HPAIVs evolve from LP progenitors after circulation in domesticated birds. They are naturally restricted to H5 and H7 subtypes. The shift of H5 and H7 viruses from LP to HP is accompanied by mutations due to the error-prone RNA-dependent RNA-polymerase (RdRp) and/or reassortment, i.e. acquisition of gene segments from other subtypes [5]. The HA protein is synthetized as a fusion-inactive precursor (HA0) which requires processing by host or bacterial proteases into HA1 and HA2 polypeptides at the proteolytic cleavage site (CS). Alteration of the CS from a monobasic to a polybasic motif after insertion of basic amino acids (aa) arginine (R) and/or lysine (K) is a major virulence factor [6,7]. The monobasic CS of LPAIV is activated by trypsin-like proteases, which are restricted to the respiratory and/or gastrointestinal tracts of birds resulting in only local infections. Human airway trypsin-like protease (HAT) and transmembrane protease serine 2 (TMPRSS2) present in human airways have been shown to cleave HA with monobasic cleavage site, however, it remains to be investigated whether orthologues proteases support HA cleavage in birds. Conversely, the polybasic CS of HPAIV is cleaved by ubiquitous, subtilisin-like proteases causing systemic infection and multiorgan dysfunction [8].

Despite carrying polybasic CS motifs, some H5 and H7 viruses exhibited low virulence in chickens [5,9]. The virulence of several of these viruses was influenced by increasing the numbers of basic aa in the CS or by additional mutations in the HA or other gene segments [10-12]. Interestingly, a few natural non-H5/H7 viruses fulfill the criteria of HPAIV. Several H10Nx viruses with monobasic CS exhibit an intravenous pathogenicity index (IVPI) higher than 1.2 resembling the HPAIVs H5/H7 [13-15]. Moreover, in August 2012, an H4N2 virus was isolated from quails in California which possessed the polybasic CS motif ^{322}PEKRRTR/G^{329} [16]. It is the only non-H5/H7 virus with 4 basic aa in the CS, which complies with the HPAIV furin-specific motif (R-X-X-R). Although the virus replicated and transmitted efficiently in chickens, it did not cause morbidity or mortality [16]. It has been reported that some H5/H7 viruses possessed “intermediate” polybasic CS which evolved stepwise to accumulate an increasing number of basic aa, due to strand slippage induced by RdRp [17] or predisposing RNA secondary structure [18], as found in the HPAIV H5N2 in Mexico in 1994 [19], H7N7 in Chile in 2002 [20], H7N7 in Canada in 2004 [21] and even the circulating H5N1 Goose Guangdong virus since 1996/1997 [22]. Also, an increase in the number of basic aa in the CS of several H5 LPAIVs with a K/R-K-K/T-R sequence, similar to the current H4N2 virus, resulted in their transformation into HP phenotypes [12,23,24]. Therefore, there is a possibility that this H4N2 virus acquires single mutations by changing T^{327} to either R or K to produce typical H5/H7 HPAIV R-X-R/K-R motifs [25]. Non-H5/H7 viruses are capable to shift to high virulence after acquisition of a polybasic CS [26,27] and other gene segments from HPAIV H5N1 [28]. However, little is known about the fitness costs which may explain the lack of expansion of non-H5/H7 HPAIV in birds.

Here, the virulence of the unique H4N2 virus in chickens was studied after the substitution of threonine at position 327 (T^{327}) to arginine (R^{327}) or lysine (K^{327}) to increase the number of basic aa to five leading to motifs ^{322}PEKRRRR/G^{329} and ^{322}PEKRRKR/G^{329} or reassortment with HPAIVs H5N1 or H7N7.

Materials and methods

Viruses, plasmids and cells

A/quail/California/D113023808/2012(H4N2) was kindly provided by Beate Crossley, the California Animal Health and Food Safety Laboratory System, Department of Medicine and Epidemiology, University of California, Davis. Plasmids containing eight gene segments of HPAIV A/swan/Germany/R65/2006(H5N1) were kindly provided by Jürgen Stech, Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institute (FLI), Greifswald-Insel Riems, Germany [10]. Moreover, plasmids containing eight gene segments of HPAIV A/chicken/Germany/AR1385/2015 (H7N7) were previously cloned [29]. pCAGGS plasmids encoding HAT and TMPRSS2 have been described previously [30].

Primary chicken embryo kidney (CEK) cells used for determining replication kinetics were prepared according to standard procedures [31]. Madin-Darby canine kidney (MDCK), MDCK type II (MDCKII), and human embryonic kidney 293T (HEK293T) cell lines were obtained from the Cell Culture Collection in Veterinary Medicine of the FLI.

Generation of plasmids and recombinant viruses

To generate the recombinant H4N2 virus (designated hereafter H4N2_wt) by reverse genetics, viral RNA was extracted using the QIAamp Viral RNA Mini Kit and transcribed into cDNA using the Omniscript RT Kit (Qiagen, Germany). All eight genomic segments of H4N2 virus were amplified by specific primers and cloned into pHW*ccdB* plasmid [32]. Using the HA encoding plasmid of H4N2_wt, three different CS motifs were generated by exchanging $T^{327}R$ or $T^{327}K$, or by insertion of a polybasic CS resembling that of HPAIV A/chicken/Italy/8/1998(H5N2) (designated hereafter H5N2- HACS) using the QuikChange II Site-Directed Mutagenesis Kit (Invitrogen, USA). Sequences of primers are available from the authors upon request.

Eight recombinant viruses (Table 1) were rescued in co-cultures of MDCKII and HEK293T cells as previously described [33]. In addition to the recombinant H4N2_wt, three recombinant H4N2 viruses carrying the HA4 with $T^{327}R$ (H4N2_$T^{327}R$), $T^{327}K$ (H4N2_$T^{327}K$) or H5N2-HACS (H4N2_H5N2-HACS*) were constructed. Moreover, three recombinant H4N1 viruses carrying seven gene segments from H5N1 and HA from H4N2_wt (H5N1_HA4), HA4_$T^{327}R$ (H5N1_HA4_$T^{327}R$) or HA4_$T^{327}K$ (H5N1_HA4_$T^{327}K$) and one H4N7 virus carrying seven gene segments from H7N7 and the HA from H4N2_wt (H7N7_HA4) were successfully generated. Furthermore, HA of H4N2_wt, H4N2_$T^{327}R$ and H4N2_$T^{327}K$ H4N2 were cloned into pCAGGS vector to increase protein expression.

Table 1: Recombinant viruses generated in this study

Virus	Cleavage site	Source of	
		HA	Other gene segment
H4N2_wt	^{322}PEKRRTR/G^{329}	H4N2	H4N2
H4N2_HA_T^{327}R	^{322}PEKRR**R**R/G^{329}	H4N2	H4N2
H4N2_HA_T^{327}K	^{322}PEKRR**K**R/G^{329}	H4N2	H4N2
H4N2_H5N2-HACS*	^{322}P**QRRRGKK**R/G^{331}	H4N2	H4N2
H7N7_HA4	^{322}PEKRRTR/G^{329}	H4N2	H7N7
H5N1_HA4	^{322}PEKRRTR/G^{329}	H4N2	H5N1
H5N1_HA4_T^{327}R	^{322}PEKRR**R**R/G^{329}	H4N2	H5N1
H5N1_HA4_T^{327}K	^{322}PEKRR**K**R/G^{329}	H4N2	H5N1

Residues written in bold indicate the point mutations or insertion compared to the wild type H4N2 virus cleavage site ^{322}PEKRRTR/G^{329}
HACS= hemagglutinin cleavage site

Virus propagation and sequencing

Recombinant viruses were propagated in the allantoic sac of 10 – 11 day-old specific pathogen free (SPF) embryonated chicken eggs (ECE) purchased from VALO BioMedia GmbH (Osterholz-Scharmbeck, Germany) according to the standard protocol of the World Organization for Animal Health (OIE) [34]. Inoculated eggs were examined daily and those with dead embryos were chilled at 4°C and allantoic fluid (AF) was collected. AF was checked by hemagglutination test using 1% chicken erythrocytes according to the OIE recommended protocol [34]. AF with a titer >16 ($4\log_2$) hemagglutination units was checked for bacterial contamination by streaking sheep blood agar at 37°C for up to 72h. Sterile AF was pooled and virus stocks were aliquoted and stored at -80°C until use. All recombinant viruses with polybasic CS, except H4N2_wt, were handled in BSL3 facilities of the FLI. Viruses were sequenced to exclude unwanted mutations by Sanger sequencing using ABI BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems, Germany). H4 aa numbering is based on the mature protein after removal of the signal peptide.

Replication kinetics

CEK and MDCK cells were infected at a multiplicity of infection (MOI) of 0.001 in 12-well plates. After one hour at 37°C and 5% CO_2, the inoculum was removed and the cells were incubated for two minutes with citric acid buffer (pH 3.0). The cells were washed twice with 1x phosphate buffered saline (PBS) and covered with Minimum Essential Medium (MEM) containing 0.2% bovine serum albumin (BSA) (MP Biomedicals, USA). Cells infected with recombinant H4N2_wt were grown in the presence or absence of 2µg/µl of N-tosyl-L-phenyalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma Aldrich, Germany). Plates were incubated at 37°C and 5% CO_2. Cells and supernatant were harvested at indicated hours

post infection (hpi) and stored at -80°C. Virus titers were determined using plaque assay as described below. The replication kinetics were run in duplicates and repeated three times. Results are expressed as average and standard deviation for all replicates.

Plaque assay

Confluent MDCKII or MDCK cells in 6-well plates were washed once with PBS and incubated with 10-fold dilutions of propagated viruses or samples for 1 hour at 37°C and 5% CO_2. Thereafter, cells were washed twice with 1xPBS and covered by semi-solid Bacto™ Agar (BD, France) with 50% MEM containing 4% BSA (MP Biomedicals, USA). All plates were incubated for 3 days at 37°C and 5% CO_2. In MDCKII cells, TPCK-treated trypsin (2 µg/ml) was added to cells infected with H4N2_wt and H4N2_H5N2-HACS*. Moreover, cell-to-cell spread of all indicated viruses in MDCK cells was studied with or without exogenous TPCK-treated trypsin. Cells were fixed using 10% formaldehyde containing 0.1% crystal violet for at least 48 hours. Plaques were counted and viral titers were expressed as plaque forming units per ml (PFU/ml). Moreover, to determine cell-to-cell spread of different viruses, the size of at least 50 plaques obtained for each virus was measured by microscopy (Eclipse Ti-S with software NIS-Elements, version 4.0; Nikon, Germany). Diameter of plaques of the H4N2_wt in the absence of trypsin was adjusted to 100%. The plaque size obtained by different recombinant viruses relative to the H4N2_wt was calculated.

Western Blot

HA cleavability was assessed in HEK293T in the presence or absence of exogenous proteases (i.e. trypsin, TMPRSS2 and HAT) as indicated using standard Western Blot procedures with few modifications [35]. Cleavage of HA of H4N2_wt, H4N2_T^{327}R and H4N2_T^{327}K in the presence or absence of 2µg/ml TPCK-treated trypsin was studied by transfecting cells with 5µg pCAGGS plasmid coding for HA of the different viruses using Lipofectamine 2000 transfection reagent (ThermoFischer Scientific, Germany). The transfected cells were incubated with MEM containing 0.2% BSA at 37°C and 5% CO2 for 24h. For TMPRSS2 and HAT, HEK293T cells (which do not express an endogenous HAT or TMPRSS2 [36]) were co-transfected with 1µg pCAGGS plasmids containing H4N2_T^{327}K as well as 10ng plasmid coding for each protease in the presence or absence of 50µM furin inhibitior MI-1148 (kindly provided by Torsten Steinmetzer, Institute of Pharmaceutical Chemistry, Philipps-University Marburg) was done as previously published [30]. After 48 h, transfected cells were harvested, washed with PBS and centrifugated at 14000g for 15min. Proteins were denatured in Laemmli buffer for 5 min at 99°C. Proteins as well as a stained protein marker were separated by discontinuous sodium dodecyl sulfate- 10% polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes using a blotting device at 25 V for 2 h and blots were blocked for 1h in 5% skim milk. For the detection of the HA protein, polyclonal specific anti-H4N2-HA2 antibodies were generated in rabbits. The β-Actin as internal control was detected using monoclonal antibodies. All blots were incubated with the primary antibodies overnight at 4°C. Bound primary antibodies were detected by the incubation of blots with peroxidase-conjugated anti-rabbit IgG for HA or anti-mouse IgG antibodies (Jackson Immuno Research, USA) for β-Actin. The immunodetection was done by chemiluminescence using Clarity™ Western ECL Substrate (BioRad, USA). Images were captured by a Bio-Rad Versadoc 4000 Molecular Imager (BioRad, USA) and Quantity One software (BioRad, USA).

Animal experiments

All animal experiments in this study were carried out according to the German Regulations for Animal Welfare in the biosafety level-3 (BSL3) animal facilities of the FLI after approval by the authorized ethics committee of the State Office of Agriculture, Food Safety and Fishery in Mecklenburg – Western Pomerania (LALLF M-V). The commissioner for animal welfare at the FLI representing the Institutional Animal Care and Use Committee (IACUC) approved all experiments.

SPF eggs from white leghorn chickens were purchased from VALO BioMedia GmbH (Osterholz-Scharmbeck, Germany) and incubated at the animal quarantine facilities of the FLI until hatch. Male and female chickens, at 6 to 8 week-old, were allocated into different groups and infected via the oculo-nasal (ON) or intravenous (IV) routes. To determine the virulence of recombinant viruses via the ON route, chickens were inoculated with 0.2 mL containing 10^5 PFU per bird (~0.1 mL in each side). One day post inoculation (dpi), sentinel chickens were added to assess bird-to-bird transmission. To determine the IVPI of indicated viruses, 10 birds were injected via the cutaneous ulnar vein with 0.1mL 1:10 diluted AF according to the OIE standard protocol [34]. All birds were observed daily for clinical signs and mortality for 10 (IV) or 14 (ON) dpi. The severity of clinical signs was calculated using pathogenicity index (PI) as recommended [34]. Briefly, healthy birds were scored with (0). Birds showing one clinical sign (e.g. ruffled feather, depression, nervous signs, diarrhea, edema, hemorrhages or cyanosis in the unfeathered parts like shanks, comb or wattle) were given score (1), and birds exhibiting at least two clinical signs were scored with (2). Dead birds were given score (3) until the end of the experiment. Severely diseased birds were euthanized and scored as dead on the next observation day. The PI was calculated using the sum of daily arithmetic means of all birds divided by ten or 14 (number of observation days) in each group. The PI value ranged from 0 (avirulent) to 3 (highly virulent).

Oropharyngeal and cloacal swabs were collected at 4 dpi using MEM containing antibiotics. Virus excretion in swab samples was determined using NucleoSpin 8/96 PCR Clean-up Core Kit (Macherey & Nagel, Germany) according to the manufacturer instructions using the TECAN Freedom EVO System (TECAN, Switzerland). After RNA extraction the viral load in the swab samples was assessed by generic real-time-reverse-transcription polymerase chain reaction (RT-qPCR) targeting the AIV Matrix gene [37]. Each RT-qPCR run included standard curves generated by serial dilutions of H4N2 or H5N1 virus. The amount of RNA was determined by plotting the CT-value of a given sample against the dilution in standard curves and expressed as viral RNA copies / ml. Results of each group are expressed as arithmetic mean and standard deviation of virus titers in oropharyngeal and cloacal swabs.

At the end of observation period, all surviving birds were euthanized by Isoflurane® (CP-Pharma, Germany) inhalation and blood was collected. Sera were tested for anti-AIV NP antibodies using ID screen Influenza Antibody Competition Multispecies kit (IDvet, Montpellier, France) according to the manufacturer recommendations.

Histopathology and immunohistochemistry

Severity of pathohistological lesions and distribution of recombinant viruses in trachea, lungs, heart, liver, pancreas, kidneys, thymus, spleen, proventriculus, gizzard, duodenum, jejunum, caecum, bursa of Fabricius and brain from at least two inoculated birds per group was analyzed at 4 dpi except for chickens inoculated with H4N2_T^{327}K which died at 2 dpi and were kept in the refrigerator. Organ samples were fixed immediately in 10% neutral buffered formalin. After

processing the samples were embedded in paraffin wax, sectioned at 2 – 4 µm, stained with hematoxylin and eosin, and screened for histopathological changes. The severity of necrotizing inflammation and lymphatic depletion was scored blind on an ordinal 0 to 3 scale: 0 = no change; 1= mild; 2 = moderate, and 3 = severe necrosis or lymphatic depletion. Following sections were used for immunohistochemistry using the avidin–biotin–peroxidase complex method (Vector Laboratories Burlingame, CA, USA) with a primary polyclonal rabbit anti-NP antibody (1:750), and a secondary biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) antibody (1:200) as described [38,39]. The distribution of NP antigen in endothelium and parenchyma was blind semiquantitatively scored on a ordinal 0 to 3 scale: 0 = negative; 1 = focal or oligofocal, 2 = multifocal, and 3 = coalescing to diffuse immunoreactive cells.

Statistics

Statistical differences for replication kinetics in CEK and MDCK cells were analyzed using ordinary one-way ANOVA with post hoc Tukey tests. Plaque size in MDCKII and MDCK cells and RT-qPCR results of oropharyngeal shedding 4 dpi were evaluated using ordinary one-way ANOVA with Bonferroni correction to H4N2_wt. A p-value < 0.05 was considered significant. All analyses were done by GraphPad Prism 8 software (CA, USA).

Results

Beside the recombinant H4N2_wt with ^{322}PE**KRR**T**R**/G^{329}, seven different viruses carrying the H4N2-HA with T^{327}R (^{322}PE**KRRRR**/G^{329}), T^{327}K (^{322}PE**KRRKR**/G^{329}) or (^{322}PQ**RRR**G**KKR**/G^{331}) with other gene segments from H4N2, HPAIV H5N1 or HPAIV H7N7 were successfully generated using reverse genetics (Table 1). Trials to generate an H4N2 carrying a typical HPAIV H5N2 HACS (^{322}PQ**RRRKKR**/G^{330}) were not successful indicating incompatibility with the H4N2_wt. After several rescue attempts, a virus was obtained with a spontaneous insertion of a glutamic acid (^{322}PQ**RRR**E**KKR**/G^{331}) in the HACS. The impact of HA mutations or reassortment on virus replication in primary chicken kidney cells and MDCK cells, cell-to-cell spread in MDCKII and MDCK cells and cleavability in the presence or absence of different proteases (i.e. trypsin, HAT or TMPRSS2) were studied. Virulence, bird-to-bird transmission, virus excretion and tropism of recombinant viruses were assessed in chickens.

The expansion of the polybasic CS had a minimal impact on virus replication in cell culture and replication of H4N2 virus was significantly increased by reassortment with H5N1 or H7N7 gene segments.

Virus replication was studied after infection of CEK (Figure 1) and MDCK (Figure 2) cells with an MOI of 0.001 in the presence or absence of exogenous trypsin. In CEK, the H4N2_wt replicated with or without trypsin reaching maximum titers at 48 hpi. The addition of trypsin slightly increased titers at 24 and 48 hpi, although it was not statistically significant ($p > 0.35$) (Figure 1). Replication of H4N2_T^{327}R and H4N2_T^{327}K with a point mutation in the HA was comparable to H4N2_wt in the presence or absence of trypsin at 8 and 24 hpi (Figure 1), while the addition of trypsin significantly increased H4N2_T^{327}R replication at 48 hpi ($p < 0.03$) (Figure 1). H4N2_H5N2-HACS* virus replication was significantly reduced (Figure 1). H7N7_HA4 carrying the HA from H4N2_wt and the other seven gene segments from HPAIV H7N7 replicated to significantly higher levels at 8, 24 and 48 hpi than H4N2_wt (Figure 1). Moreover, the three viruses carrying seven gene segments from HPAIV H5N1 replicated to

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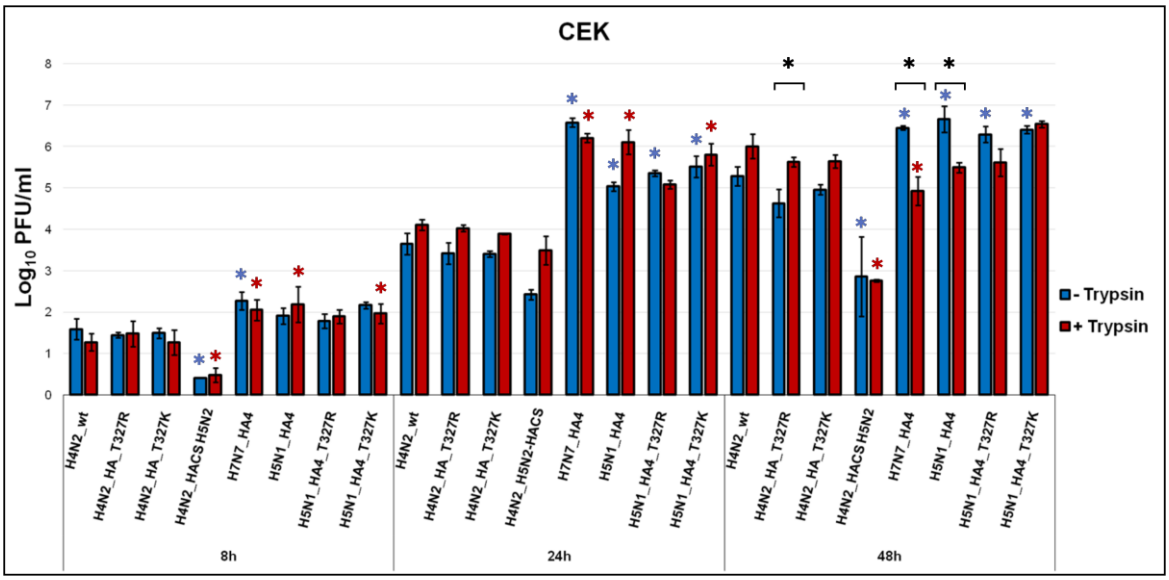
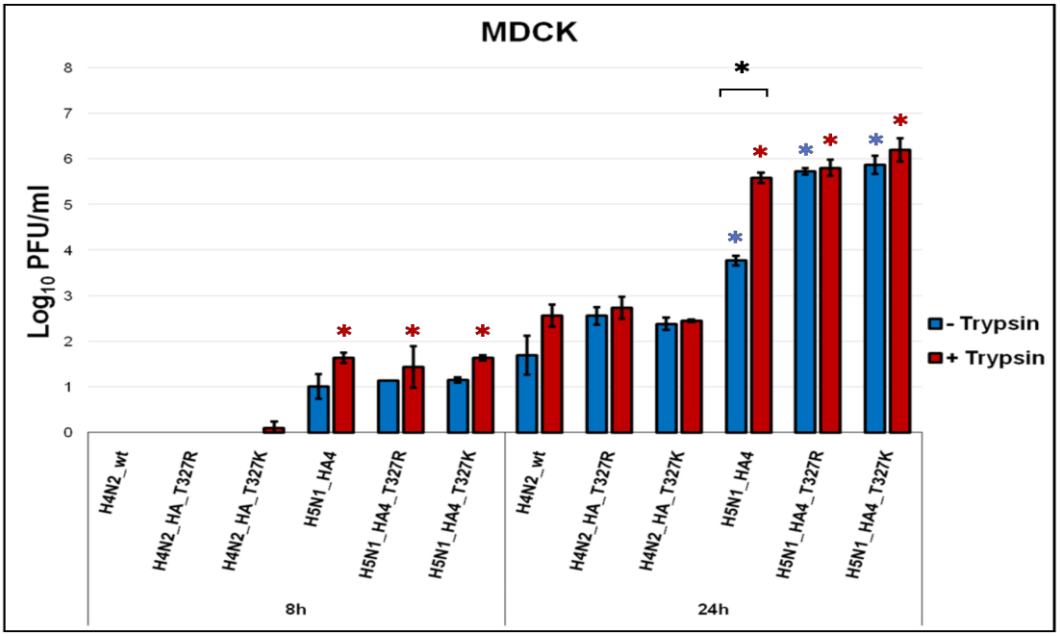
significantly higher titers than H4N2_wt particularly at 24 hpi (Figure 1). In MDCK cells, H4N2 viruses carrying authentic HA, HA_T^{327}R or HA_T^{327}K didn't grow at 8 hpi without trypsin and only H4N2_HA_T^{327}K at very low titer with trypsin (Figure 2). H4 viruses carrying gene segments from H5N1 were able to replicate trypsin-independently but replication increased after the addition of trypsin. At 24 hpi, all viruses replicated without trypsin while viruses carrying H5N1 gene segments replicated at significantly higher titers than H4N2 viruses with or without T^{327}R/K. Furthermore, trypsin increased replication of H5N1_HA4 to similar levels to H5N1_T^{327}R/K (Figure 2). Together, the replication of H4N2 viruses was enhanced by the addition of trypsin particularly in MDCK cells and reassortment with HPAIV H5N1 or H7N7 gene segments in CEK.

Figure 1: Replication kinetics of recombinant viruses in chicken embryo kidney cells (CEK)



Replication kinetics in CEK cells at indicated time points after infection with H4N2 viruses with variable HACS or with H7N7 and H5N1 gene segments with (T+) or without (T-) trypsin. Titration was done in MDCKII cells and the results are shown as mean ± standard deviation Log$_{10}$ PFU/ml. Asterisks indicate significant difference (P value <0.05). Blue and red asterisk indicate significant differences compared to H4N2_wt without or with trypsin, respectively.

Figure 2: Replication kinetics of recombinant viruses in Madin-Darby canine kidney cells (MDCK)



Replication kinetics in MDCK cells at indicated time points after infection with H4N2 viruses with variable HACS or H5N1 gene segments with (T+) or without (T-) trypsin. Titration was done in MDCKII cells and the results are shown as mean ± standard deviation Log_{10} PFU/ml. Asterisks indicate significant difference (P value <0.05). Blue and red asterisk indicate significant differences compared to H4N2_wt without or with trypsin, respectively.

Cell-to-cell spread of H4N2 virus was significantly increased by reassortment with H7N7 or H5N1 containing HA4_T^{327}R/K.

Cell-to-cell spread was studied by infecting MDCKII and MDCK cells with different virus dilutions for 3 days. In MDCKII cells, all viruses were tested without trypsin except for H4N2_wt and H4N2_H5N2-HACS*. The H4N2_wt virus produced plaques in MDCKII in the presence or absence of trypsin, although addition of trypsin significantly increased the size of plaques (Figure 3 panel A). The spread of H4N2_T^{327}R/K or H5N1_HA4 from cell-to-cell was comparable to the H4N2_wt in the absence of trypsin. H4N2_H5N2-HACS* produced significantly smaller plaques without trypsin (data not shown). However, in the presence of trypsin plaques were larger than those produced by H4N2_wt (Figure 3 panel A). The plaque size induced by H4N2_T^{327}R or H4N2_T ^{327}K significantly increased by 52% and 125% when combined with other H5N1 gene segments (Figure 3 panel A). In MDCK cells, infection with all viruses was done in the presence or absence of trypsin. The addition of trypsin increased the plaque size produced by all viruses. Viruses carrying H5N1 gene segments produced larger plaques than H4N2_wt particularly when grown in medium containing trypsin (Figure 3 panel B). Taken together, the expansion of the polybasic CS had a minimal impact on virus spread in cell culture and replication of H4N2 virus was significantly increased by reassortment with H5N1 and/or addition of trypsin.

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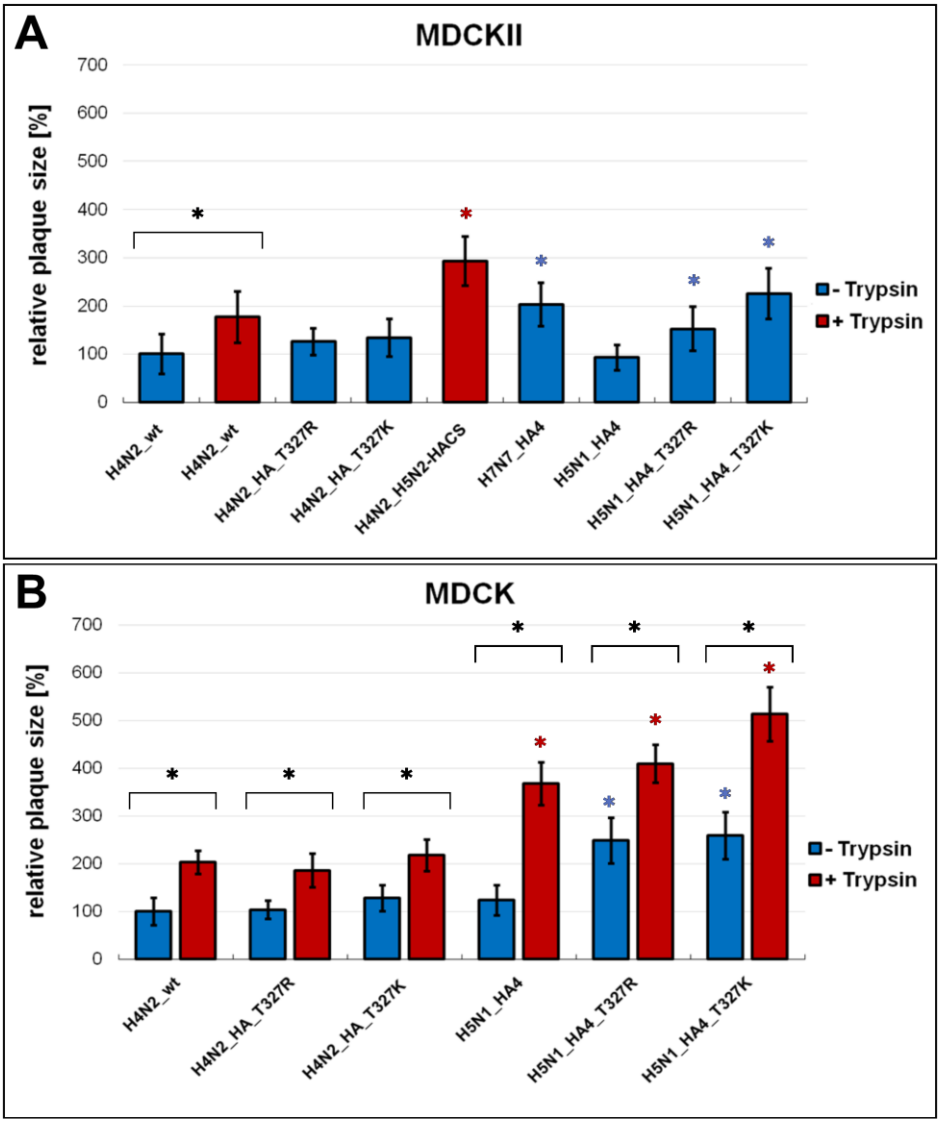
Figure 3: Cell-to-cell spread of MDCKII and MDCK cells



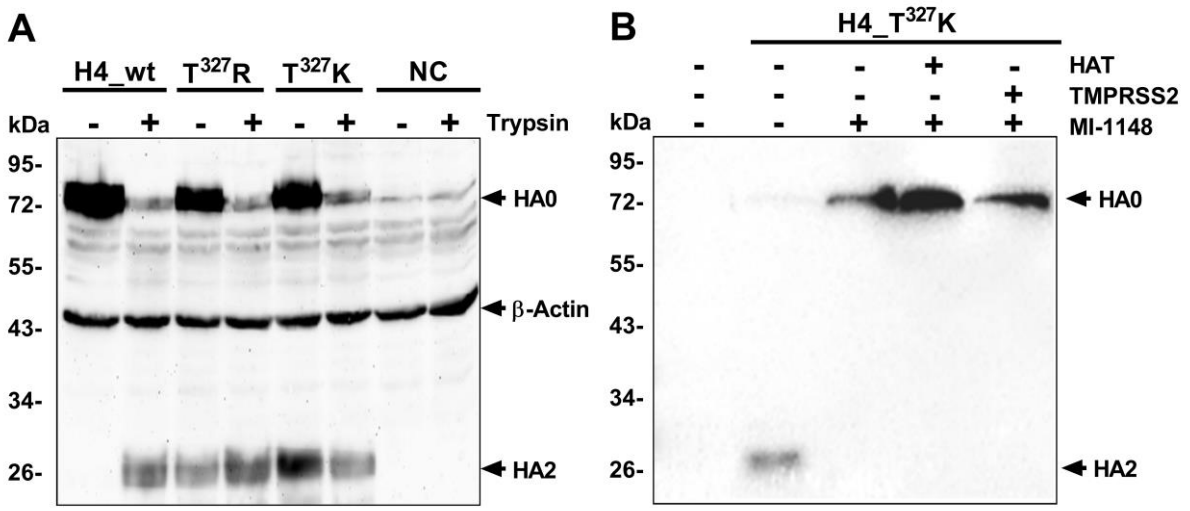
Cell-to-cell spread was assessed by measuring 50 to 100 plaques in MDCKII (A) or MDCK (B) cells with (T+) or without (T-) trypsin. In MDCKII (A), trypsin was added only to H4N2_wt and H4N2_H5N2-HACS. The latter virus didn't produce plaques without trypsin (data not shown). Results expressed as mean and standard deviation relative to plaque size of H4N2_wt in the absence of trypsin. Asterisks indicate significant difference (P value <0.05); blue and red asterisk indicate significant differences compared to H4N2_wt without or with trypsin, respectively.*

T^{327}R/K enabled partial trypsin-independent activation of the HA by endogenous furin-like protease.

To determine the impact of expansion of CS on HA cleavage activation, HEK293T cells were transfected with pCAGGS plasmids containing HA_wt, T^{327}R or T^{327}K with or without trypsin. The wild-type HA was only cleaved in the presence of trypsin. HAs with T^{327}R/K were partially cleaved in the absence of trypsin but addition of trypsin increased cleavability (Figure 4 panel A). Furthermore, to get insights into the proteolytic activation of T^{327}K in the absence of trypsin,

HEK293T cells were co-transfected with plasmids encoding HAT and TMPRSS2 in the presence or absence of the furin-inhibitor MI-1148. In the absence of exogenous protease $T^{327}K$ was cleaved. The cleavage of HA was inhibited using MI-1148. HAT and TMPRSS2 failed to activate HA_$T^{327}K$ (Figure 4 panel B). The expression of TMPRSS2 and HAT in HEK293T cells was confirmed using Western Blot (data not shown). Altogether, $T^{327}R/K$ enabled partial activation of the HA in the absence of trypsin by yet to be identified endogenous furin-like protease.

Figure 4: Cleavability of the HA after transfection of HEK293T cells.



Cleavability of the HA of H4N2_wt or HA with R^{327}or K^{327} in presence or absence of exogenous trypsin tested with Western Blot using β-Actin as internal controls (A). The cleavability of HA of H4N2_$T^{327}K$ in HEK293T cells by TMPRSS2, HAT or furin-like proteases in the presence or absence of the MI-1148 furin inhibitor. No HA2 bands were detected indicating lack of activation of HA0 by TMPRSS2 and HAT (B). NC refers to negative control; naïve cells without transfection or infection.

The expansion of the cleavage site alone was not enough for exhibition of high virulence after ON inoculation and reassortment with HPAIV H5N1 genes was essential.

After ON inoculation, chickens challenged with H4N2_wt, H4N2_$T^{327}R$, H4N2_$T^{327}K$, H4N2_H5N2-HACS* and H7N7_HA4 and contacts did not show any clinical signs with PI of 0 (Table 2). All primarily challenged birds in these groups seroconverted at the end of the experiment, except one chicken inoculated with H4N2_H5N2-HACS* (Table 2). While all sentinels in-contact to H4N2_wt, H4N2_$T^{327}R$, and H7N7_HA4 ON-inoculated chickens seroconverted, only 1/4 and 0/4 sentinel birds co-housed with chickens ON-inoculated with H4N2_$T^{327}K$ or H4N2_H5N2-HACS* seroconverted (Table 2), respectively indicating poor bird-to-bird transmission. Moreover, H5N1_HA4 caused transient mild to moderate clinical signs without mortality (PI= 0.5) and all inoculated and contacts seroconverted (Table 2). All chickens inoculated with H5N1_HA4_$T^{327}R$ or H5N1_HA4_$T^{327}K$ died within 4 dpi with MDT values of 3.8 and 2 days, and PI values of 2.4 and 2.7, respectively. Furthermore, 3/4 and 4/4 contact birds died within 8 and 4 dpi, respectively (Table 2).

Table 2: Results of clinical examination after challenge of chickens with different recombinant viruses in this study

Virus	Oculonasal					IVPI
	Inoculated chickens			Contact chickens		
	PI[1]	Mortality (MDT; range)	SC	Mortality (MDT; range)	SC	
H4N2_wt	0.0	0/6 (n.a.; n.a.)	4/4	0/3 (n.a.; n.a.)	3/3	n.d.
H4N2_HA_T^{327}R	0.0	0/6 (n.a.; n.a.)	4/4	0/4 (n.a.; n.a.)	4/4	0.0
H4N2_HA_T^{327}K	0.0	0/6 (n.a.; n.a.)	4/4	0/4 (n.a.; n.a.)	1/4	0.6
H4N2_H5N2-HACS*[2]	0.0	0/6 (n.a.; n.a.)	3/4	0/4 (n.a.; n.a.)	0/4	0.1
H7N7_HA4_wt	0.0	0/6 (n.a.; n.a.)	4/4	0/4 (n.a.; n.a.)	4/4	0.3
H5N1_HA4_wt	0.5	0/6 (n.a.; n.a.)	4/4	0/4 (n.a.; n.a.)	4/4	2.1
H5N1_HA4_T^{327}R	2.4	6/6 (3.8; 3 – 4)	n.a.	3/4 (6.0; 4 – 8)	1/1	n.d.
H5N1_HA4_T^{327}K	2.7	6/6 (2.0; 2)	n.a.	4/4 (3.5; 3 – 4)	n.a.	2.8

[1]PI= pathogenicity index, Mortality Rate= number of dead birds/total number of birds per group, MDT= mean death time and range of days with mortality after inoculation or adding the sentinel birds, SC= Seroconversion using NP-specific ELISA showing number of positive birds/total examined, IVPI= intravenous pathogenicity index, n.a. = not applicable, n.d. = not done.

[2]HACS= hemagglutinin cleavage site

After IV infection, none of H4N2_T^{327}R injected chickens showed morbidity or mortality with IVPI values of 0. H4N2_T^{327}K exhibited moderate virulence with an IVPI of 0.6 where moderate to severe neurological disorders (e.g. torticollis, opisthotonus, paresis) starting at 9 dpi were observed in 7 out of 10 injected chickens. The IVPI values of H4N2_H5N2-HACS* and H7N7_HA4 were 0.1 and 0.3, respectively because some chickens developed transient mild depression after injection. Conversely, 8/10 and 10/10 chickens injected IV with H5N1_HA4 or H5N1_HA4_T^{327}K died with IVPI 2.1 or 2.8, respectively (Table 2). These findings indicate that H5N1 gene segments, in addition to mutations in the HACS, are essential for exhibition of high virulence of H4N2 virus. T^{327}K and H5N2_HACS compromised virus transmission as indicated by lower number of contact birds with AIV antibodies.

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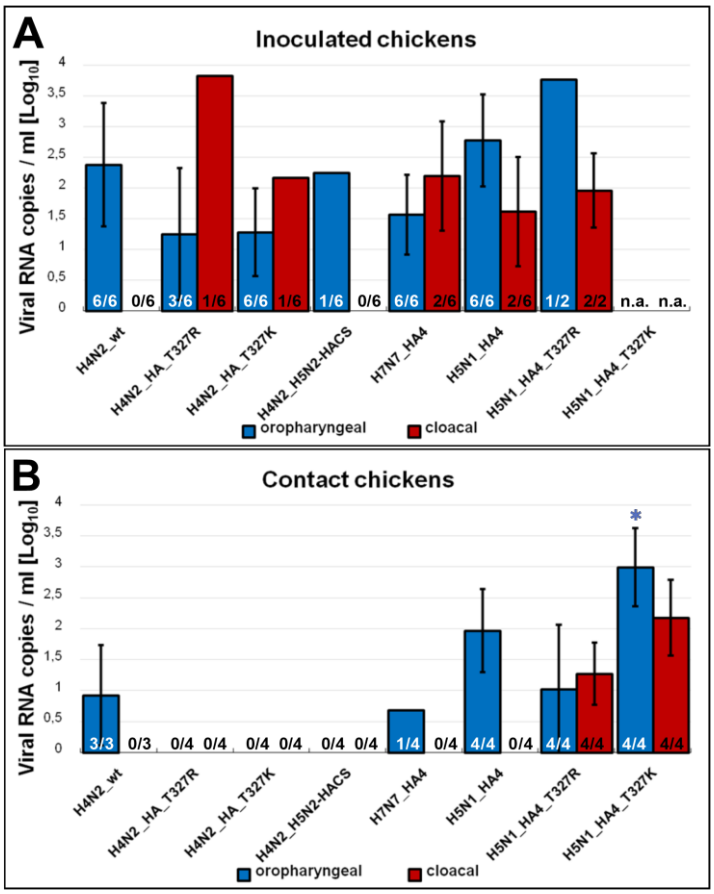
Peer-reviewed version available at Int. J. Mol. Sci. 2020, 21, 2353; doi:10.3390/ijms21072353

Virus excretion in inoculated and in-contact chickens was reduced by $T^{327}R/K$ and increased by acquiring HPAIV H5N1 genes.

The H4N2_wt was detected at 4dpi in oropharyngeal, but not in cloacal, swabs in all inoculated and contact birds (Figure 5). H4N2_T^{327}R and H4N2_T^{327}K were excreted in 3/6 and 6/6 in oropharyngeal swabs, respectively and only in 1/6 cloacal swabs in inoculated birds (Figure 5 panel A). Both viruses were not detected in swabs in contact birds further indicating the negative impact on virus transmission (Figure 5 panel B). Likewise, H4N2_H5N2-HACS* RNA was only detected in the oropharyngeal swabs taken from 1/6 inoculated chickens indicating insufficient replication and bird-to-bird transmission (Figure 5 panels A and B). Moreover, H7N7_HA4 RNA was detected in oropharyngeal and cloacal swabs in 6/6 and 2/6 inoculated birds, respectively and only 1/4 contact bird excreted virus in the oropharyngeal swabs (Figure 5). H5N1_HA4 RNA was detected in 6/6 and 2/6 oropharyngeal and cloacal swabs of inoculated birds, respectively and all contact birds excreted virus in oropharyngeal swabs (n=4/4) but not in cloacal swabs (n=0/4) (Figure 5 panels A and B). H5N1_HA4_T^{327}R was detected in oropharyngeal (n=1/2) and cloacal (n=2/2) swabs (Figure 5 panel A). Because H5N1_HA4_T^{327}K killed all inoculated birds within 2 days, it was not possible to collect swabs at 4dpi in this group. Both H5N1_HA4_T^{327}R and H5N1_HA4_T^{327}K were excreted from all contact birds (Figure 5 panel B). H5N1_HA4_T^{327}K had significantly higher titers in the oropharyngeal swabs compared to H4N2_wt (Figure 5). In summary, mutations in the CS alone compromised virus excretion from inoculated and in-contact chickens. The reassortment with segments from HPAIV H5N1 increased virus excretion in oropharyngeal and cloacal swabs.

Figure 5: Virus excretion from oropharyngeal and cloacal swabs of inoculated and sentinel chickens.

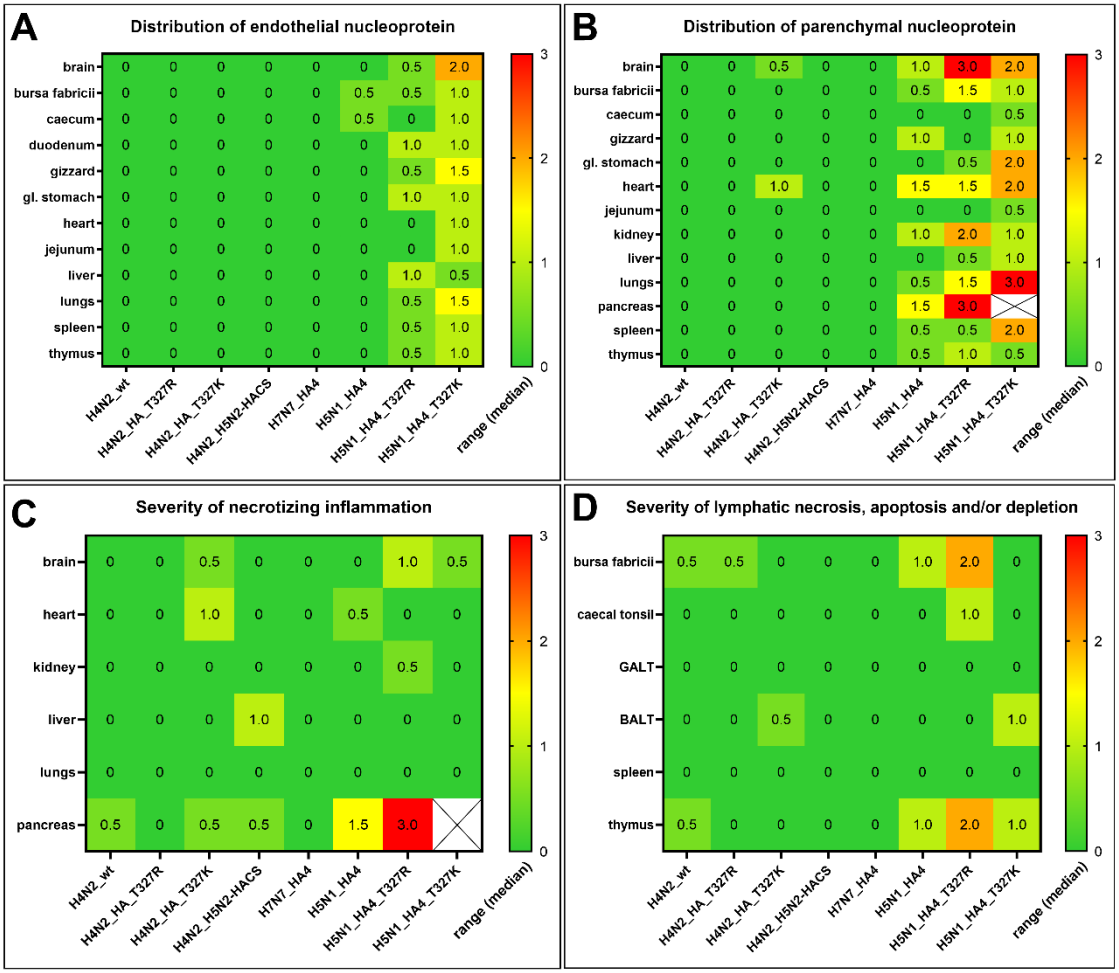


Virus excretion in oropharyngeal and cloacal swabs in inoculated (A) and contact (B) birds was determined by RT-qPCR targeting the M gene. Shown is the average ± standard deviation of viral RNA copies/ml and number of positive birds/total examined. Samples were collected at 4dpi from all surviving birds. n.a.= not applicable because all birds inoculated with H4N2_T^{327}K died at 2 dpi.

Mutation T^{327}R/K expanded the organ tropism of LPAIV H4N2 and reassortment with HPAIV H5N1 significantly increased the distribution and severity of lesions.

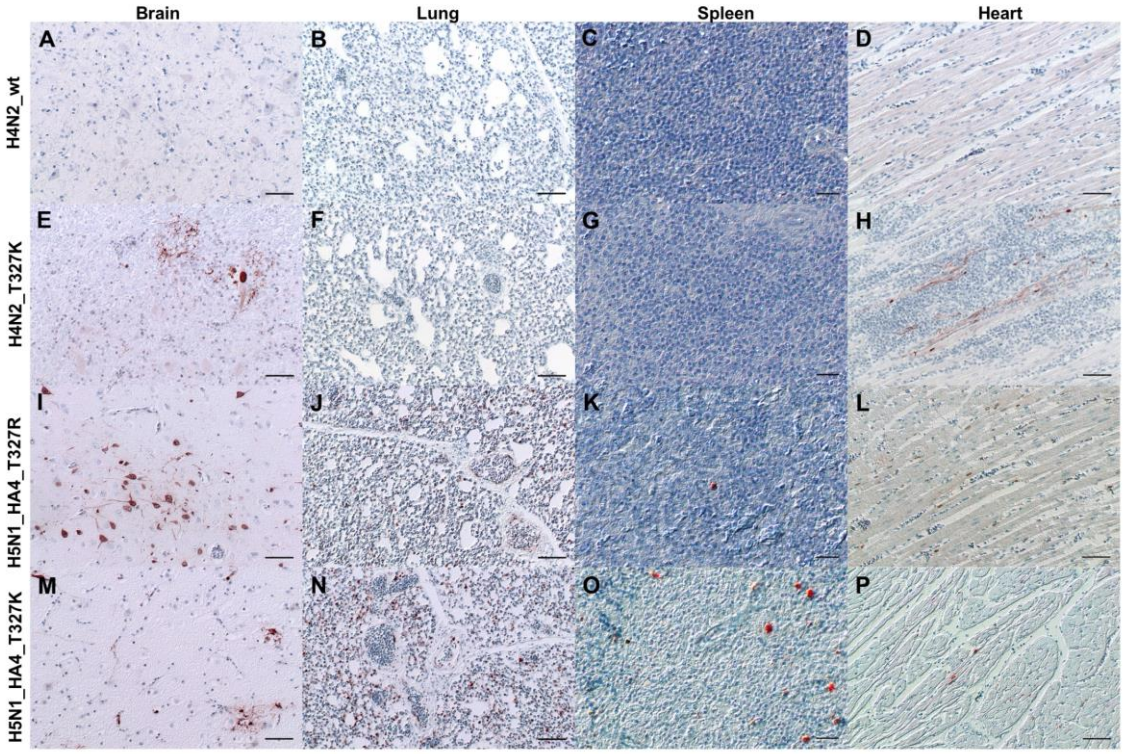
To determine the distribution of viruses in different tissues, organs of at least two inoculated chickens per group were subjected to histopathological and immunohistological examination for the detection of influenza NP antigen. There was no detectable antigen in the endothelium or parenchyma of any organ in birds inoculated with H4N2_wt, although mild, subacute, necrotizing pancreatitis and lymphatic depletion in the thymus and bursa of Fabricius were observed (Figure 6). Likewise, NP antigen was not detectable in the endothelium or parenchyma of any organ in birds inoculated with H4N2_T^{327}R. However, one out of two birds inoculated with H4N2_T^{327}K had multifocal antigen distribution in the myocardium with mild, acute, focal to oligofocal necrotizing myocarditis as well as focal to oligofocaldistribution in neuroglial cells with mild, acute, focal to oligofocal, necrotizing polioencephalitis (Figures 6 and 7). The distribution of H4N2_T^{327}K was more widespread than that of H4N2_T^{327}R (Figure 6). Similar to H4N2_wt infection, NP was not detected in the endothelium or parenchyma of any organ in birds inoculated with H4N2_H5N2-HACS* or H7N7_HA4 (Figure 6 A and B). The reassortment with HPAIV H5N1 remarkably increased the distribution of the virus in different tissues. One out of two birds inoculated with H5N1_HA4 showed coalescing NP-antigen-positive cells in the heart and pancreas, multifocal distribution in the kidney, gizzard and brain, and focal to oligofocal distribution in the parenchyma of thymus, lung, spleen and bursa as well as in the endothelial cells in cecum and bursa. This bird showed moderate to severe lymphoid depletion with tingible body macrophage hyperplasia in the thymus and mild lymphoid depletion in the bursa. Also, severe, acute, necrotizing pancreatitis and subacute, necrotizng myocarditis were observed. The NP of H5N1_HA4_T^{327}K was detected in the endothelial and parenchymal cells of almost all organs (Figure 6 panels A and B) and the intensity ranged from median scores of 0.5 in the hepatic endothelium as well as in thymus, jejunum and caecum parenchyma to 3.0 in the lung parenchyma. Likewise, H5N1_HA4_T^{327}R was detected in the endothelium and parenchyma of almost all organs of at least one chicken, except for endothelial cells in jejunum, heart and caecum and gizzard parenchyma. The maximal distribution for this virus was in the pancreas parenchyma and brain tissue with a score of 3.0. Remarkably, H5N1_HA4_T^{327}R induced higher lymphatic depletion score in the thymus, bursa, cecal tonsils and bronchus-associated lymphoid tissues (BALT) compared to H5N1_HA4_T^{327}K and H5N1_HA4. In conclusion, reassortment with HPAIV H5N1 significantly increased the distribution and severity of lesions. The distribution of viruses carrying T^{327}K was remarkably higher than viruses carrying T^{327}R (Figures 6 and 7); except for lymphoid depletion.

Figure 6: Distribution of avian influenza virus nucleoprotein in organs of inoculated birds and severity of lesions.



Distribution of NP antigen in endothelial (A) and parenchymal (B) cells as well as the severity of necrotizing inflammation (C) and lymphatic depletion (D) in the affected organs of inoculated birds scored from 0 to 3.0 (green to red color). Results are shown as median score of two birds. Samples were collected at 4 dpi for all chickens, except H5N1_H4_T^{327}K inoculated birds which died at 2 dpi and kept in the refrigerator until 4 dpi. Pancrease samples with white cells and X symbol in panels B and C were not tested because of insufficient quality for evaluation.

Figure 7: Distribution of avian influenza virus nucleoprotein in selected organs of inoculated birds.



Distribution of influenza NP in brain (A, E, I, M), lung (B, F, J, N), spleen (C, G, K, O) and heart (D, H, L, P) of inoculated chickens of selected viruses at 4 dpi (except for H5N1_H4_T^{327}K) as detected by immunohistochemistry using primary polyclonal rabbit anti-NP A/FPV/Rostock/34 antibody (1:750) and a secondary biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) antibody (1:200). 3-amino-9-ethyl-carbazol (red-brown); hematoxylin counterstain (blue); Nomarski contrast; Bars A,B,D,E,F,H,I,J,L,M,N,P = 20 µm. Bars C,G,K,O = 50 µm.

Discussion

Wild birds are the reservoir for LPAIV. HPAIV evolve from LP progenitors of H5 or H7 subtype after the acquisition of a polybasic CS which is specific in each HPAIV. In 2012, an H4N2 virus with polybasic CS ^{322}PEKRRTR/G^{329}, closely related to H4N2 viruses with monobasic CS ^{322}PEKTR/G^{329} from wild birds in the USA, was isolated from a commercial quail flock in California [16]. In our study, the acquisition by H4N2 virus of a 'classical' polybasic CS either by mutation of T^{327} to K^{327} or R^{327}, or the substitution by an H5N2-like HACS did not improve virus replication or spread in the absence of trypsin. Conversely, H4N2_H5N2-HACS* was highly trypsin dependent as shown by low virus titers in cell culture and cell-to-cell spread. A similar example is the trypsin-dependent Pennsylvanian H5N2/1983 virus with a polybasic CS which was efficiently cleaved by furin-like enzymes only after the insertion of further basic aa or removal of a glycosylation site in the vicinity of the CS [40]. Interestingly, the current H4N2 virus has potential glycosylation sites in the vicinity of CS in the HA1 [16], resembling the H5N2 in Pennsylvania, which may sterically hinder the

cleavability by different proteases [41,42]. Moreover, we showed that furin-like protease(s) can also cleave the HA of H4N2_T^{327}K in transfected HEK293T cells. This was independent of HAT and TMPRSS2 which activate some viruses with monobasic CS [43] and some H9N2 viruses with monobasic VSSR/G, dibasic RSSR/G or tribasic RSRR/G cleavage site motifs [42]. Interestingly, these viruses were not activated by furin without further insertion of basic aa at the CS despite matching the minimal consensus sequence [26,44]. Furthermore, Wong, et al. [16] showed that the growth or plaque formation of the wild type H4N2 virus in MDCK cells was trypsin dependent. However, the current reverse-engineered H4N2 virus and derivatives induced plaques with variable size in MDCKII or MDCK cells and replicated in CEK without the addition of trypsin. It has been reported that MDCKII and CEK cells have matriptase which is not present in the MDCK cells [42]. Matriptase activated H9N2 AIVs with R-X-X-R or R-X-R-R motifs [42], similar to the HACS of H4N2 viruses generated in this study. Moreover, some LPAIVs (e.g. H6N1 and H7N7) were able to replicate in MDCK, MDCKII and/or CEK cells without exogenous trypsin [27,45]. In addition to the unidentified endogenous proteases in these cells, an impact of proteases in the allantoic fluid in virus stocks on activation in different cells cannot be excluded [46].

Successful replication of an AIV in poultry is a prerequisite for progressive adaptation including efficient bird-to-bird transmission and high virulence [47]. We show here that the expansion of the authentic polybasic CS by insertion of K^{327}, R^{327} or substitution by an H5N2-like HACS was detrimental for H4N2 virus excretion and bird-to-bird transmission. Therefore, the negative impact of additional basic aa in the CS on virus replication and transmission in chickens probably precludes their emergence in nature. Moreover, the increased number of basic aa in the presence of other gene segments from H4N2 did not result in HP phenotype after ON or IV infections resembling H5/H7 viruses [23,45]. In other studies, the insertion of a polybasic CS conferred high virulence to a low-pathogenic H6N1 virus (IVPI= 1.4) [27] but not an H3N8 virus [48]. Importantly, high virulence of the current H4N2 virus was only conferred after reassortment with gene segments from HPAIV H5N1. These findings emphasize the role of other gene segments, in addition to the polybasic CS, in the evolution of HPAIV [10,11,49]. H9N2 with polybasic CS and gene segments from HPAIV H5N1 exhibited a low level HP phenotype (IVPI= 1.23) [26]. In contrast, H2N5, H4N6, H8N4 and H14N3 viruses exhibited high virulence after the acquisition of an H5N2-polybasic CS and other gene segments from HPAIV H5N1 [28]. Remarkably, high virulence was not observed after reassortment of the H4N2 HA with HPAIV H7N7. We have recently shown that the HA gene of this HPAIV H7N7 is the main determinant of virulence in chickens [29]. Conversely, for the current HPAIV H5N1, in addition to the polybasic CS, the deletion within the NA stalk domain (which is present in the quail H4N2 virus [16]) and the presence of autologous polymerase genes were important for exhibition of high virulence in chickens [10]. Similarly, it has been shown that PB2, PB1 and NP affect high virulence of HPAIV H5N1 in chickens [11].

Moreover, it is known that presence of a polybasic CS increases dissemination of HPAIV H5/H7 in different organs causing multiorgan dysfunction and death of the bird [6,14,25,27,48]. We showed here that a polybasic CS alone was not enough for unrestricted organ tropism and that other gene segments were required particularly to invade the endothelium to vital organs like the brain. This may indicate that the quail virus is less adapted to chicken cells than the panzootic Goose/Guangdong-like H5N1 virus. Apart from the activation of HA by cellular proteases, other gene segments can influence influenza virus activation and replication as well. The NA enhanced the cleavability of the HA of WSN H1N1 and subsequently the neurovirulence of the virus in mice [50,51]. Also, the M2 protein protects the HA from premature conformational changes increasing the stability of influenza viruses

[52]. Therefore, it is important to further determine which specific gene segment(s) of H5N1 support the HP phenotype of H4N2 virus.

Another finding was that $T^{327}K$ was advantageous over $T^{327}R$. It increased the plaque size, virulence in chickens after IV injection, tropism and excretion from inoculated birds particularly when combined with H5N1 gene segments. This may indicate cleavage-activation of this CS motif (^{322}PEKRR**K**R/G^{329}) by additional or more specific furin-like proteases. Some proteases have different preferences for K and R at different positions [53]. For example, lysine in position P2 can greatly enhance the processing efficiency of furin-like enzymes. In one study, 20 (52%) out of the 38 cleavage motifs comply with furin specific sequences were R–X–K–R and 11 (29%) were R–X–R–R giving preferences for lysine over arginine in this position [54]. Intriguingly, the majority of HPAIVs H5/H7 possessed lysine at position P2 [22], resembling the $T^{327}K$ in this study which may support our assumption.

In conclusion, the insertion of additional basic aa in the polybasic CS compromised H4N2 replication and transmission in chickens which were restored by reassortment with HPAIV H5N1. Therefore, due to the negative impact of the polybasic CS on virus fitness, sudden evolution of HPAIV H4N2 in nature is unlikely. Although it remains speculative, the evolution of natural HPAIV H4N2 will require firstly reassortment with HPAIV H5N1 like gene segments to achieve higher fitness and followed by mutations in the HA to enable wide protease-activation. The fitness cost of the artificially induced polybasic CS as indicated by poor transmission and replication of H4N2 viruses carrying K^{327}, R^{327} or H5N2-like HACS after ON inoculation may be a strong limiting factor for evolution of non-H5/H7 HPAIVs. Such viruses may occur as a result of error-prone RdRp activity, but they are less fit than the wild type H4N2 viruses and most likely will be eliminated from the quasispecies.

Acknowledgments
The authors are grateful to Dajana Helke, Nadine Bock and Diana Kruhl for laboratory technical assistance, to Günter Strebelow for his assistance in sequencing, to Dr. Christine Fast, Bärbel Hammerschmidt, Felix Winter, Frank Klipp, Mathias Jan, Harald Manthei, Doreen Fiedler, Bärbel Berger, Thomas Moeritz and Ralf Henkel for their support in the animal experiments and to Silvia Schuparis for histotechnological preparations. Dr. Jürgen Stech thankfully provided the plasmids of H5N1 virus used in this study.

Author contributions
EMA, JV, EBF and TCM conceived and designed the study. MG, DS, AHS and EMA conducted the animal experiments. BC provided the H4N2 virus. RU and OIF conducted the histopathological analysis. MG conducted the statistical analysis. MG and EBF conducted the in-vitro characterisation. EMA wrote the manuscript. All authors read and approved the final manuscript.

Funding
This work was supported by grants from the Deutsche Forschungsgemeinschaft DFG AB 567/1-1 and DFG VE780/1-1 and Collaborative Research Centre SFB1021. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interests
The authors declare no conflict of interest

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