Genetic determinants for virulence and adaptation of avian influenza virus subtype H4N2 in chickens and subtype H10N7 in mammals

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To my lovely daughters

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aa	amino acid
AIV	avian influenza virus
cDNA	complementary DNA
CEK	chicken embryonated kidney
cRNA	complementary RNA
EE	early endosome
GS	N-glycosylation site
HA	hemagglutinin
HA0	inactive, pre-mature hemagglutinin
HA1	hemagglutinin subunit 1
HA2	hemagglutinin subunit 2
HACS	hemagglutinin cleavage site
HAT	human airway trypsin-like protease
HEK	human embryonic kidney
HP	highly pathogenic
HPAIV	highly pathogenic avian influenza virus
IAV	influenza A virus
IV	intravenously

IVPI	Intravenous Pathogenicity Index
LBM	live bird market
LE	late endosome
LP	low pathogenic
LPAIV	low pathogenic avian influenza virus
M1	matrixprotein 1
M2	matrixprotein 2
mCS	monobasic cleavage site
MDCK	Madin-Darby Canine Kidney
mRNA	messenger RNA
NA	neuraminidase
NEP	nuclear export protein
Neu5Ac	N-acetylneuraminic acid
NP	nucleoprotein
NS1	non-structural protein 1
OIE	World Organization for Animal Health
ORF	open reading frame
PA	polymerase acidic
PB1	polymerase basic subunit 1
PB2	polymerase basic subunit 2
PCR	polymerase chain reaction
pCS	polybasic cleavage site
pGS	potential glycosylation site
QU/CA12	A/Quail/California/D113023808/2012 (H4N2)
RBS	receptor binding site
RdRp	viral RNA-dependent RNA polymerase
SA	sialic acid
TGN	trans-Golgi network
TMPRSS2	transmembrane serine protease 2
vRNA	viral RNA
vRNP	viral ribonucleoprotein

1. Introduction

1.1. Influenza A virus (IAV)

1.1.1. Taxonomy

Influenza A viruses (IAVs) belong to the family *Orthomyxoviridae*, which also contains the genera Influenza Virus B, C, D, Infectious Salmon Anaemia, Quaranjavirus and Thogotovirus. The classification is based on variations of the nucleoprotein (NP) and the matrix protein 1 (M1) (Hause et al., 2014; ICTV, 2020).

In both, human and veterinary medicine, IAVs are one of the leading health concerns (Capua and Alexander, 2010; Paget et al., 2019). Their evolution in different host species led to the formation of distinct IAV lineages. Hence, there are three main avian lineages with viruses from Eurasia, North America and Oceania. Similarly, host-specific lineages for human, classical swine and equine IAVs exist. Moreover, in pigs, two cross-host lineages, the Eurasian avian-like swine and the swine-origin human pandemic H1N1 2009 lineage, as well as bat viruses are known (Tong et al., 2013; Xu et al., 2011).

Besides the NP classification, IAVs are subdivided into 18 hemagglutinin (HA) and 11 neuraminidase (NA) subtypes due to the variable antigenic and genetic properties of the HA and NA, respectively (Fouchier et al., 2005; NCBI, 2021; Tong et al., 2013). Furthermore, the H1-H18 HA and N1-N11 NA subtypes are classified into two distinct phylogroups (Gamblin and Skehel, 2010).

1.1.2. Virion structure

Influenza A viruses are polymorphic with a host-derived double bilayer lipid membrane and mainly spherical or rarer filamentous shape. While spherical particles are around 100 nm in diameter, filamentous virions have a length of around 300 nm (Bouvier and Palese, 2008; Bruce et al., 2012). The core of the virion contains the segmented viral RNA (vRNA) genome. Each RNA segment appears as a viral ribonucleoprotein (vRNP) complex consisting of the RNA interacting with the polymerase complex and many copies of NP (Elton et al., 1999; Gavazzi et al., 2013; Nayak et al., 2009; Noda et al., 2006). Virions are packed in a 7+1 arrangement where one vRNP complex in the center is surrounded by the other seven vRNPs in a circular manner (Eisfeld et al., 2015; Noda et al., 2006). Furthermore, the interior contains M1, the nuclear export protein (NEP) and probably the non-structural protein 1 (NS1), which was a long time believed to be located only in host cells during infection (Bouvier and Palese, 2008; Hutchinson et al., 2014). M1 locates beneath the viral envelope surrounding the particle core and plays an essential role in the architecture and morphology of IAVs by linking the vRNPs with the three transmembrane proteins (Elleman and Barclay, 2004; Enami and Enami, 1996; Rossman and Lamb, 2011). Besides the integral membrane matrix protein 2 (M2), the two glycoproteins HA and NA stud the virus envelope like

spikes (Bouvier and Palese, 2008) (Figure 1). Interestingly, the ratio of transmembrane proteins is different and can vary between virus strains. Accordingly, one virus particle exhibits 14 to 68 M2 proteins, while the ratio to HA ranges between 1:10 to 1:100 (Zebedee and Lamb, 1988). Likewise, the HA:NA ratio is about 2:1 to 8:1 and varies among viruses too (Webster et al., 1968)

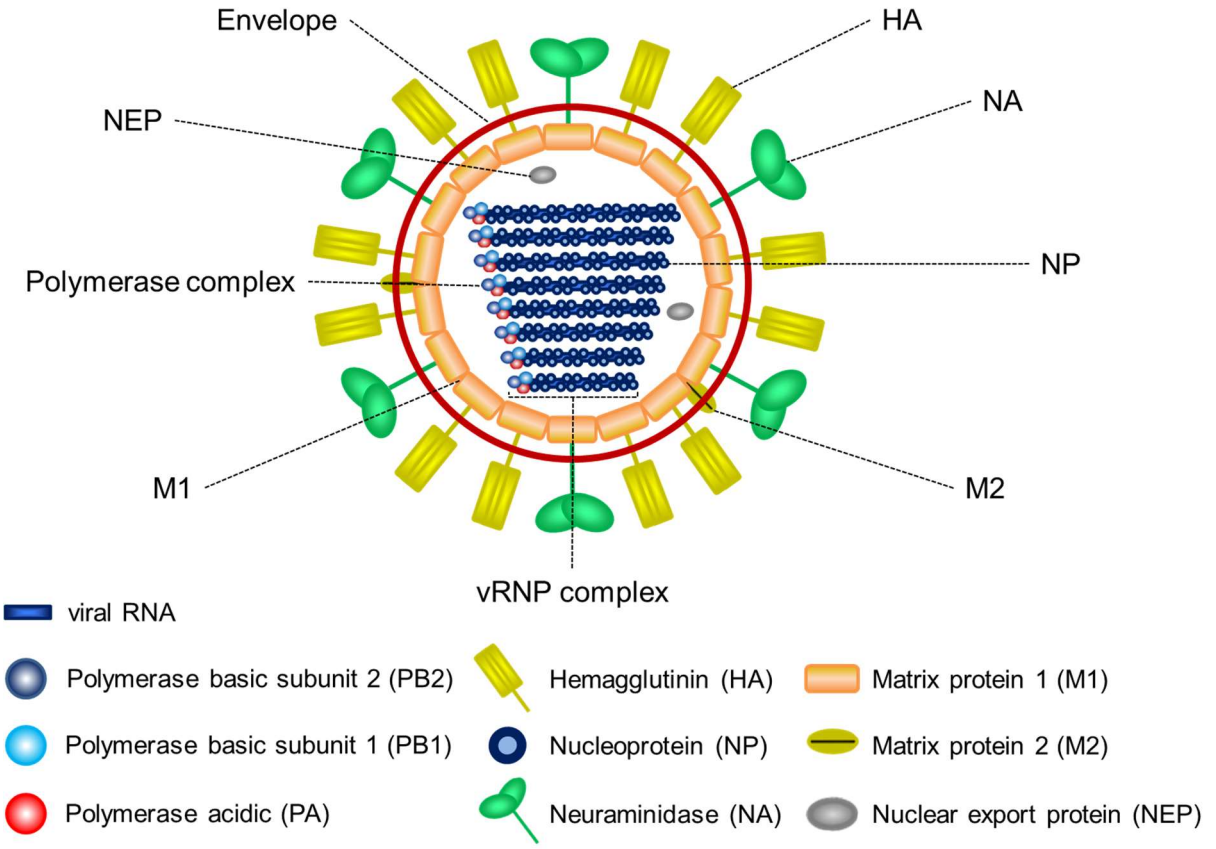

Figure 1: Schematic structure of an influenza A virus. Structural proteins and segmented viral RNA are depicted within the viral ribonucleoprotein (vRNP) complex.

1.1.3. Genome structure

The segmented RNA genome of IAVs consists of eight distinct single-stranded molecules with negative polarity and a total molecular size of around 13.5 kb (Bouvier and Palese, 2008; Calder et al., 2010). These RNA segments consist of a non-coding region with highly conserved terminal ends, and adjacent segment-specific nucleotides flanking the open reading frames (ORFs) of the different genes (Goto et al., 2013; Hoffmann et al., 2001; Muramoto et al., 2006). The terminal ends of each segment contain a packaging signal, which plays a crucial role for segmental interaction and thus genome selection and packaging (Hutchinson et al., 2010; Muramoto et al., 2006). Each segment codes at least for one structural protein. Moreover, segments differ in size from 2341 kb to 890 kb and are numbered from 1 – 8 in the order of decreasing length (Bouvier and Palese, 2008; Ghedin et al., 2005). The three largest segments 1 - 3 encode the viral polymerase subunits, which are formed by polymerase basic subunit 2 (PB2), polymerase basic subunit 1 (PB1) and polymerase acidic (PA) proteins (Guu et al., 2008; Stevaert and Naesens, 2016).

NP as part of the vRNP complex is synthesized through the translation of segment 5. Segment 4 and 6 encode HA and NA, respectively. M1 and NEP are synthesized from segment 7 and 8 (Bouvier and Palese, 2008). Additionally, segment 7 also encodes M2 and its subtype-specific isoform M42 produced by mRNA splicing (Wise et al., 2012). Segment 8 further encodes for NS1 (Hutchinson et al., 2014). Beside the structural proteins, the majority of IAVs express non-structural proteins PB2-S1, PB1-F2; PB1-N40; PA-X; PA-N155; PA-N182; NS3 or NSP by using alternative ORFs or ORF frameshifts, initiation sites, truncations or even the RNA minus strand of the segment (Baez et al., 1980; Clifford et al., 2009; Jagger et al., 2012; Muramoto et al., 2013; Vasin et al., 2014; Wise et al., 2009; Yamayoshi et al., 2016).

1.1.4. Viral proteins

All structural proteins are necessary for productive virus replication. The three largest proteins, PB2, PB1 and PA, forming the polymerase complex, act synergistically for a successful transcription of vRNA into translatable mRNA to synthesize viral proteins (Eisfeld et al., 2015). Besides the encapsidation of vRNA, NP has an essential role for vRNA transport to the nucleus and transcription initiation (Cros et al., 2005; O'Neill et al., 1995). NS1 serves as an interferon antagonist to inhibit the host immune response upon infection (Hale, 2014; Marc, 2014), whereas NEP mediates the nucleocytoplasmic export of nascent vRNPs (Gao et al., 2014; Neumann et al., 2000; O'Neill et al., 1998). Besides its structural and linking properties, M1 plays a crucial role for the vRNP release into the cytoplasm and the virus assembly (Bui et al., 1996). In contrast, the M2 homotetramer forms an ion channel to acidify the interior of the virion (Sugrue and Hay, 1991; Wharton et al., 1994). The transmembrane glycoprotein NA facilitates virus spread due to mucin breakdown in the respiratory tract, virus motility, prevention of virus particle aggregation and finally, the release of the virus from the cell membrane due to a highly conserved sialidase enzyme pocket (McAuley et al., 2019; Shtyrya et al., 2009). By contrast, HA is essential for receptor-mediated binding, endocytosis and fusion of the viral and host membrane prior to vRNP release into the cytoplasm. (Blijleven et al., 2016; Lakadamyali et al., 2004).

1.1.5. Replication cycle of IAVs

The replication of IAVs is a complex process where viruses hijack the transport and replication machinery of the host cell. Therefore, the replication cycle can be divided into the attachment phase, virus entry, uncoating and vRNP release, RNA transcription and translation, posttranslational modification, assembly and finally, the virus release (Figure 2).

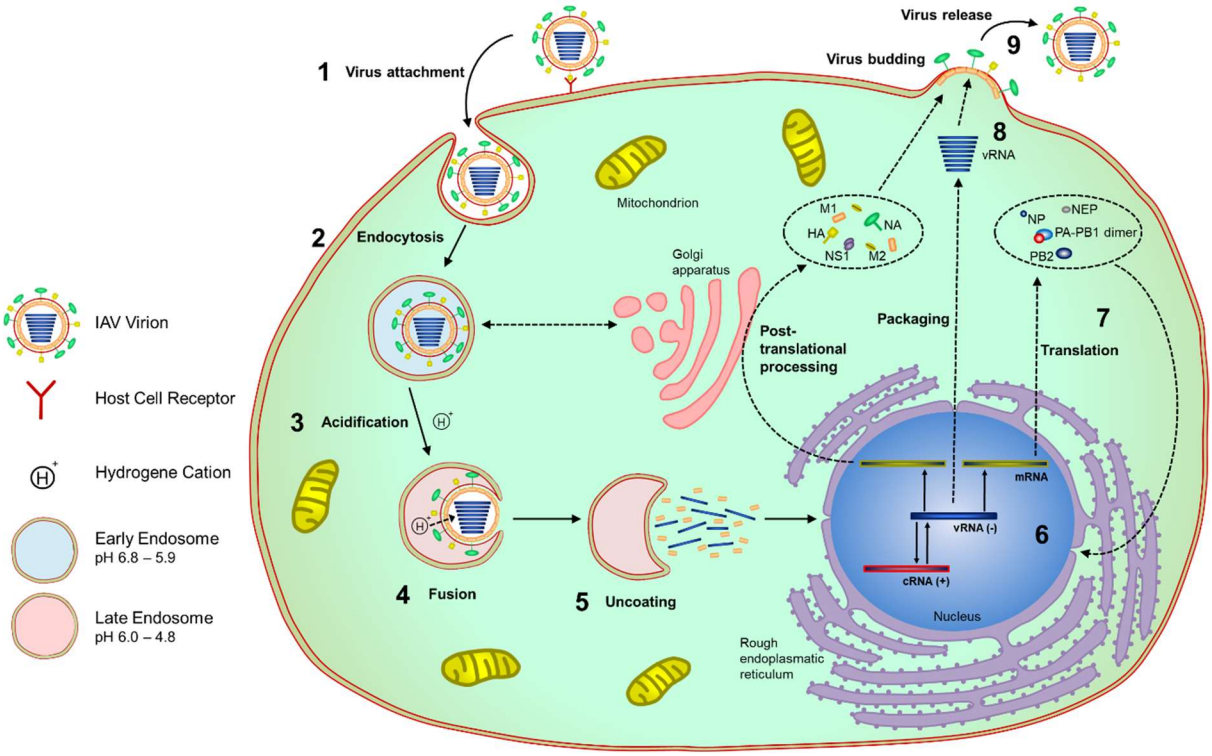

Figure 2: Replication cycle of influenza A viruses. The virion binds via hemagglutinin (HA) to receptors on the host cell during the attachment phase (1) and gets internalized by endocytosis (2). The virion gets acidified during endosome maturation (3) and consequently, the cleaved HA forces the fusion of viral and endosomal membranes (4). Following the fusion pore formation, the viral ribonucleoprotein-complexes are released into the cytoplasm during the uncoating step (5). After the transport to the nucleus, the vRNA gets replicated and transcribed (6). Then the mRNA gets translated and posttranslational processing takes place (7) prior to assembly and budding of viruses at the apical membrane (8). Finally, nascent virions are released via neuraminidase cleavage from the host cell surface (9).

In the attachment phase, the HA of parental virions binds to receptors on the host cell surface (Varki, 2008), further described in paragraph 1.1.6.1. The viral entry phase starts by receptor-mediated internalization (Matlin et al., 1981; Yoshimura et al., 1982). IAVs hijack different host mechanisms usually needed for the uptake of fluids and macromolecules, predominantly clathrin-mediated endocytosis and macropinocytosis but also clathrin and calveolae independent pathways (De Conto et al., 2011; de Vries et al., 2011; Lakadamyali et al., 2004; Rossman et al., 2012; Rust et al., 2004). The internalization ends in an early endosome (EE) containing the virus particle (Lakadamyali et al., 2004). Subsequently, the transport to the perinuclear region, mediated by dynamic actin, takes place. During a rapid dynein-mediated translocation to the perinuclear region, the EE starts to mature and acidify by fusion with vesicles from the trans-Golgi network (TGN) and from late (matured) endosomes (LEs) (Lakadamyali et al., 2003). The acidification continues and the EE evolves to an LE during an intermitted microtubule-mediated movement in the perinuclear region. Thereby, the vesicle pH drops from 6.8 – 6.1 to 6.0 – 4.8 using proton pumps (Huotari and Helenius, 2011; Perez and Carrasco, 1994). The M2 ion channel enables the proton influx from the LE into the virion and subsequently acidifies the virus interior (Sugrue and Hay, 1991; Wharton et al., 1994).

The low pH forces the activated HA trimers into a conformational change to merge the viral and endosomal membranes so that they eventually form a stable fusion pore (Blijleven et al., 2016; Maeda and Ohnishi, 1980; Melikyan et al., 1993). A closer look at the membrane fusion is given in paragraph 1.1.6.2. Moreover, the drop in pH causes dissociation of M1 from the viral envelope and a weaker or even lost binding to NP, necessary for the subsequent release of vRNPs into the cytosol (Bui et al., 1996). Therefore, IAVs hijack the host cell aggresome formation and disassembly machinery (Banerjee et al., 2014; Fontana et al., 2012; Su et al., 2013).

Influenza viruses belong to the few RNA viruses that replicate in the nucleus. The transport of the released vRNPs is mediated by the nuclear localization signal encoded by NP (Cros et al., 2005; O'Neill et al., 1995). Likewise, NP facilitates the vRNP-interaction with the nuclear pore complex. However, a successful transfer into the nucleus requires cellular proteins like karyopherin (importin) (Babcock et al., 2004). Once in the nucleus, the negative sense vRNA is transcribed by the viral RNA-dependent RNA polymerase (RdRp) into complementary positive-sense RNA (cRNA), which serves as the template for new vRNA copies (Fan et al., 2019). Furthermore, the viral RdRp transcribes the vRNA into mRNA, which is used to translate the viral proteins (Engelhardt et al., 2005; Lukarska et al., 2017). To initiate synthesis of the viral mRNA, cRNA must be capped and polyadenylated to mimic a host mRNA, which gets translated in the cytoplasm by the translation machinery (Eisfeld et al., 2015). To that end, a process called “cap snatching” takes place. The cap-binding domain of PB2 binds to the 5´ cap structure of host-derived pre-mRNA, which is then cut 10 – 13 nucleotides downstream by the endonuclease activity of PA. This “stolen” cap primes the PB1 induced transcription of viral mRNA (Dias et al., 2009; Reich et al., 2014). Furthermore, each gene segment contains a conserved uracil stretch at the 5 prime end to generate the 3 prime mRNA polyadenylation signal. For encapsidation and export of nascent vRNPs, NP, NEP and M1 are transported back into the nucleus after translation in the cytoplasm (Cros et al., 2005; Gabriel et al., 2011; Gao et al., 2014; Neumann et al., 2000; Noton et al., 2007).

Proteins are synthesized as monomers, but the majority mature to homo- or heteromultimers with two, three or four subunits connected by cysteine residues via covalent disulfide bonds (Air, 2012; Elton et al., 1999; Kemble et al., 1992; Marc, 2014; Sugrue and Hay, 1991; Varghese et al., 1983). Several post-translational modifications are essential for the viral replication. Translated IAV proteins are modified by, e.g., phosphorylation, palmitoylation, ubiquitination, sumoylation and glycosylation (Hutchinson et al., 2012; Kirui et al., 2016; Tate et al., 2014; Veit and Schmidt, 1993; Way et al., 2020). The latter modification is defined by the oligosaccharide transfer to asparagine (N) site chains with an amino acid (aa) motif of N-X-S/T, where X can be any aa except proline (Tate et al., 2014). It is well established that only HA and NA possess N-glycosylation sites (GS), which vary among different viruses/subtypes (Shtyrya et al., 2009; Tate et al., 2014).

Virus assembly and budding occur at the apical plasma membrane. While integrated membrane proteins HA, NA and M2 are directed by apical sorting signals (Ohkura et al., 2014), nascent vRNP

complexes are translocated via NEP (O'Neill et al., 1998; Paterson and Fodor, 2012). It is assumed that vRNP-NEP complexes bind M1, which interacts with the cytoplasmatic tail of HA, NA and M2 (Noton et al., 2007; Zhang et al., 2000). Finally, every nascent virion must contain all structural proteins and one copy of each vRNA segment for assembly and budding (Chou et al., 2012; Fournier et al., 2012). The newly-formed viruses are still bound to the cell surface due to sialic acid interaction with the newly formed HA protein. For virus release, enzymatic cleavage of the sialic acids by NA is required (McAuley et al., 2019; Shtyrya et al., 2009).

1.1.6. Hemagglutinin of IAVs

The homotrimeric rod-shaped hemagglutinin is a type I transmembrane protein and the major glycoprotein of IAVs (Steinhauer, 1999). Hemagglutinin is synthesized at the rough endoplasmic reticulum and transported via the endoplasmic reticulum and the TGN to the plasma membrane for assembly with other structural proteins (DuBois et al., 2011; Skehel and Wiley, 2000). During the secretory pathway, protein folding, homotrimerization and posttranslational modifications like glycosylation and palmitoylation take place (Braakman et al., 1991; Daniels et al., 2003; Tate et al., 2014; Veit and Schmidt, 1993). Each monomer is synthesized as the naïve but inactive precursor HA0 with a molecular size of around 75 kDa, which is activated by proteolytic cleavage at the HA cleavage site (HACS) into HA1 (55 kDa) and HA2 (25 kDa) subunits (Steinhauer, 1999; Zhirnov et al., 2002). While HA1 exclusively forms the globular head domain, which carries the most immunogenic epitopes and the receptor binding site (RBS), HA2 forms the central part of the stem domain. It contains the transmembrane domain as well as the hydrophobic fusion peptide necessary for membrane fusion and two antiparallel α-helices responsible for the coiled-coil structure of the homotrimer. (Steinhauer, 1999; Wilson et al., 1981) (Figure 3A). A conserved arginine (R) (rarer lysine (K)), glycine (G) bond forms the HACS and is located in the fusion loop that protrudes from the HA stem at the membrane-proximal third of the molecule (Skehel and Wiley, 2000; Steinhauer, 1999). Depending on the HACS motif or structures adjacent to it, cleavage takes place during (1) maturation in the TGN, (2) on the host cell surface or (3) during release or income of virions (Bottcher-Friebertshauser et al., 2010; Skehel and Wiley, 2000; Stieneke-Grober et al., 1992).

1.1.6.1. Receptor binding

The receptor binding site is located at the tip of the globular head and is composed of proximal and flanking loop regions and a short helix at the membrane distal edge (Figure 3B). Several residues like Tyr98, Trp153, His183 and Tyr195 are conserved among all HA subtypes and essential for multivalent binding to non-O-acetylated N-acetylneuraminic acids (Neu5Ac) (Gamblin and Skehel, 2010). This member of the sialic acid (SA) family, present as terminal residue of glycoproteins and glycolipids on the cell surface, constitutes the basic determinant of the IAV host range depending on the SA linkage to galactose (Byrd-Leotis et al., 2017). Cells in the upper respiratory tract of humans predominantly exhibit SA in α2,6 linkage, while the lower respiratory tract possesses both,

α2,3-SA and α2,6-SA, conformations (Shinya et al., 2006). In comparison, mice express more α2,3-SA (Ning et al., 2009). However, the variations among bird species are broader. For instance, while ducks have mainly α2,3-SA, terrestrial poultry like chickens and turkeys additionally exhibit α2,6 linkage (Kimble et al., 2010). Accordingly, human and mammalian viruses preferentially bind α2,6 linked SA, while avian and equine viruses infect cells predominantly with Neu5Ac in α2,3 conformation (Connor et al., 1994). Thus, the SA distribution on cells, particularly in the upper respiratory and intestinal tracts, can be barriers for interspecies transmission (de Graaf and Fouchier, 2014; Suzuki et al., 2000; Webster et al., 1992). Interestingly, some viruses have a dual receptor specificity and few animals, known as “mixing vessels,” possess both α2,3-SA and α2,6-SA in the upper respiratory tract. Since α2,6 orientation of SA is a prerequisite for an efficient transmission to mammals, mixing vessels like pigs and quails can support the generation of human-adapted and pandemic viruses (Ha et al., 2001; Scholtissek, 1990; Wan and Perez, 2006; Zhang et al., 2013). Notably, several mutations in the RBS and adjacent residues can alter the preference of the receptor type (Table S1) and are discussed in paragraph 1.2.5.1.

1.1.6.2. Membrane fusion and stability

After endocytosis, virus particles are transported into endosomes with a steadily increasing acidic interior (Lakadamyali et al., 2003). Due to the decreased pH, the fusion of the viral envelope and cell membrane starts. Critical residues in the HA get protonated which results in an irreversible conformational change of the cleaved HA (Bullough et al., 1994; Han et al., 2001; White and Wilson, 1987). Accordingly, the N-terminal fusion peptide of HA2 is inserted into the endosomal membrane using a hydrophobic pocket and interacts with lipid acryl chains (Chen et al., 1999; Tsurudome et al., 1992). Afterwards, large conformational changes and re-organization of helices and the interhelix loop of the HA stem bring the membranes in direct proximity to form a “fusion site” (Yang et al., 2020) (Figure 3C and 3D). Then, the HA tilts and forces the outer leaflets to interact (Tatulian et al., 1995). However, a “fusogenic unit” requires at least three homotrimers to induce lipid curvature. The curvature results in a hemifusion stalk which eventually collapses and forms a stable fusion pore (Blijleven et al., 2016; Danieli et al., 1996; Melikyan et al., 1993). The optimal fusion pH varies among different strains (pH 4.8 – 6.2). The HA stability impacts the virus-host specificity, pathogenicity and the infectivity in *ex vivo* models (Mair et al., 2014; Russell, 2021) and is further discussed for avian and mammalian hosts in the paragraphs 1.2.3.1 and 1.2.5.1.

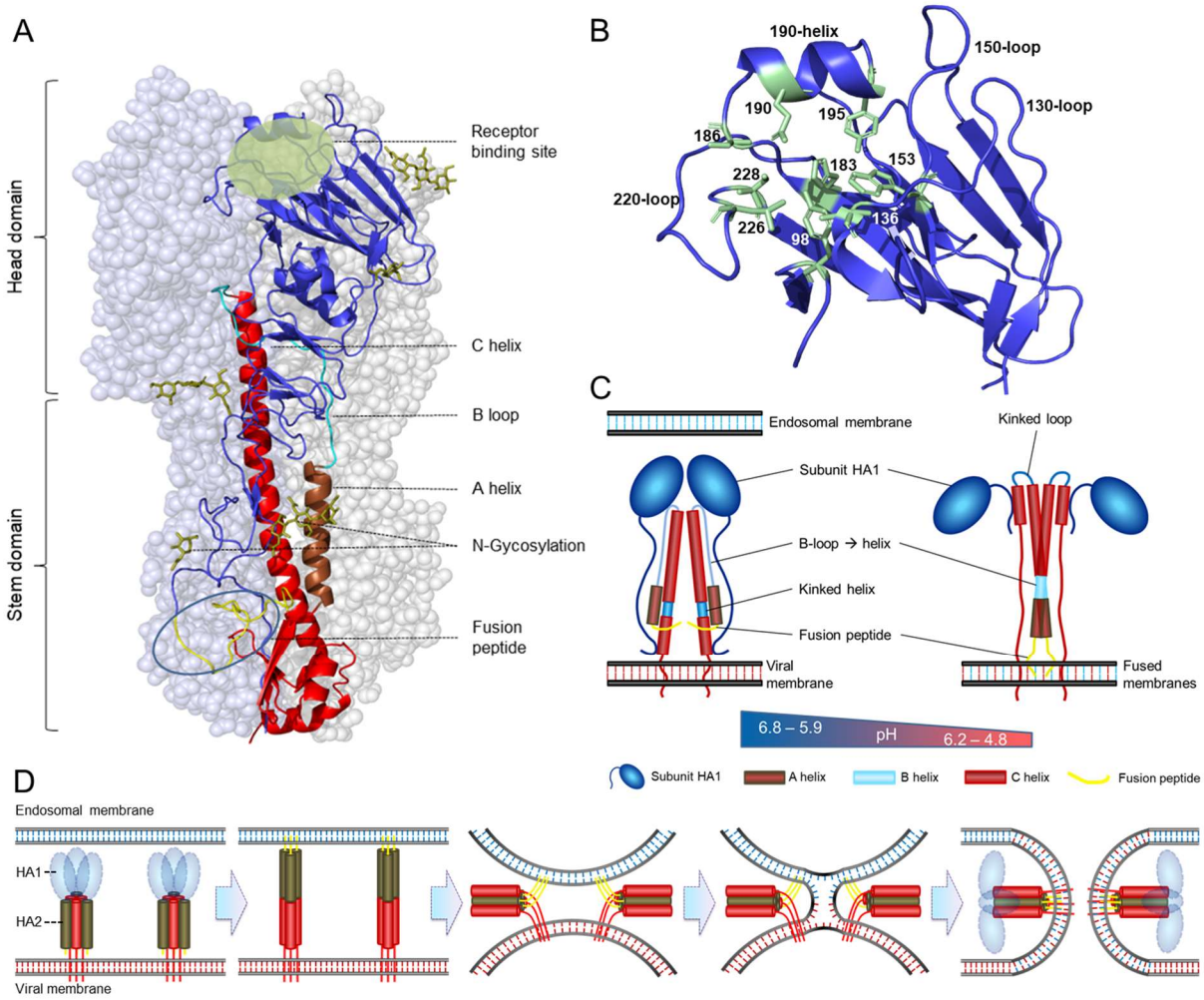

Figure 3: Structure and conformational change of hemagglutinin. (A) A hemagglutinin homotrimer with one monomer depicted in cartoon style, comprising N-glycosylations and highlighted locations of the receptor binding site and the fusion peptide. (B) A detailed view of the receptor binding site with framing tertiary structures and critical residue positions for receptor binding and switch in host preference– *modified from Byrd-Leotis et al. (2017)*. (C) A schematic depiction of the conformational changes of cleaved hemagglutinin (shown as dimer for better visualization) as a result of the drop in pH during acidification– *modified from Fontana et al. (2012)*. (D) The progress of HA2 mediated membrane fusion and formation of a fusion pore (HA1 is not shown in the intermediate steps for better visualization) - *modified from Skehel and Wiley (2000)*. The HA1 subunit is depicted in blue, while the HA2 is shown in brown and red. The fusion peptide is presented in yellow.

1.1.7. Antigenic drift, antigenic shift and recombination

The evolution and natural gain of functions of IAVs occur by three distinct ways changing the viral genome (Shao et al., 2017). Firstly, the “antigenic drift” is defined as single mutations in the viral genome caused by the error-prone vRNA RdRp lacking a proofreading function. This can lead to aa substitutions and subsequently possible alterations of the protein structure and biological functions (Ahlquist, 2002; Chen and Holmes, 2006). Such mutations can be acquired due to antivirals, vaccination pressure or spread to other species, and may confer antiviral resistance, immune evasion and increased replication in the new host (Carrat and Flahault, 2007; Landolt and Olsen, 2007). Interestingly, mutations are often observed in the HA to, e.g., mediate immune

evasion or modulate receptor binding specificity. Besides point mutations, swapping whole gene segments can occur during co-infections of single host cells by different IAVs (Reid and Taubenberger, 2003). Such “reassortments” may result in “antigenic shifts” and the possible emergence of pandemic viruses. Accordingly, the pandemic viruses from 1918/1919, 1957, 1968 and 2009 evolved by reassortment and comprised segments of IAVs derived from different hosts (Mostafa et al., 2018). Additionally, non-homologous and homologous “recombination” can occur by shuffling parts of host cell ribosomal RNA or IAV gene segments into IAV genes, mainly in the HA (Kapczynski et al., 2013; Orlich et al., 1994; Suarez et al., 2004). Hence, the insertion of, e.g., basic aa into the HACS enabled the transition of some low pathogenic (LP) to highly pathogenic (HP) avian influenza viruses (AIVs) in terrestrial birds (Suarez et al., 2004), discussed in detail in paragraph 1.2.3.1.

1.1.8. Reverse genetics, cloning and mutagenesis

The term “reverse genetics” defines the generation and modification of recombinant organisms to initiate and investigate distinct phenotypes. For IAVs, it means the generation of recombinants from cloned complementary DNA (cDNA) to investigate wild-type viruses, mutants and/or reassortants and to produce vaccines (Govorkova et al., 2006; Neumann, 2020; Neumann et al., 2005). Thereby the breakthrough was to use the eukaryotic RNA polymerase I for the transcription of viral RNA (Neumann et al., 1994). Beside its location in the nucleus and the high expression in dividing cells, RNA polymerase I initiates and terminates transcription at defined promotor and terminator sequences and can synthesize rRNA of >20 kb, enough for IAV segments with 0.9 – 2.3 kb. Moreover, transcripts do not possess 5´-cap or 3´-poly(A) structures and resemble vRNA (Neumann, 2020). Neumann et al. (1999) cloned cDNAs of all segments individually between a human RNA polymerase I promoter and a mice terminator sequence. The eight plasmids were then transfected into highly susceptible human embryonic kidney (HEK) 293T cells, together with four expression plasmids, which encode for NP and the polymerase complex to initiate virus replication and transcription. This approach resulted in the first *de novo* synthesis of an IAV from cloned cDNA in a 12-plasmid system (Neumann, 2020; Neumann et al., 1999). By using HEK 293T and Madin-Darby Canine Kidney (MDCK) cell co-cultures, a higher virus rescue efficacy was reached. While the first cell line (i.e., HEK 293T) facilitates transfection and virus generation, the second cell line (MDCK) supports efficient replication of the rescued virus (Hoffmann et al., 2000).

An improvement of the 12-plasmid system is the bidirectional reverse genetics system, which reduces the required plasmids from 12 to 8, and thus increases transfection efficacy and virus rescue. In the bidirectional system, the pol I promotor–cDNA–terminator transcription cassette in negative sense orientation is flanked by a truncated RNA polymerase II promotor from the human cytomegalovirus and from the polyadenylation signal of the gene encoding bovine growth hormone in positive sense orientation. Accordingly, the newly designed pHW2000 plasmid vector gives the ability to transcribe authentic vRNA and mRNA to synthesize viral proteins and rescue viruses from

eight plasmids simultaneous (Hoffmann et al., 2000). Moreover, further modifications on the pHW2000 vector enable the efficient, rapid and less laborious rescue of influenza viruses (Stech et al., 2008).

To modify the cDNA by point-mutations, insertions and/or deletions, several site-directed mutagenesis methods based on PCR were established (Aiyar et al., 1996; Bachman, 2013; Heda et al., 1992; Hemsley et al., 1989; Weiner et al., 1994). For implementation, primers carrying the mismatch oligonucleotides are designed either back-to-back or overlapping (Aiyar et al., 1996; Hemsley et al., 1989). A commercial kit developed by Agilent (Stratagene) for the overlapping design is available (Agilent, 2015; Zheng et al., 2004) where resulting plasmids with or without mutations can be directly transformed into competent *E.coli* cells.

1.2. Avian influenza virus (AIV)

1.2.1. Classification and assessment of pathogenicity

Avian influenza viruses contain 16 hemagglutinin (H1-H16) and 9 neuraminidase (N1-N9) subtypes (Alexander, 2015). According to the Terrestrial Code of the World Organization for Animal Health (OIE), they can be divided into two pathotypes depending on the severity of clinical signs and genetic properties of the HA protein (OIE, 2021a, b). While low pathogenic avian influenza viruses (LPAIVs) induce mild or no clinical disease, highly pathogenic avian influenza viruses (HPAIVs) (“fowl plague”) cause severe clinical signs with up to 100% morbidity and mortality. Only viruses from subtypes H5 or H7 exhibit high virulence in poultry. Such HPAIVs derive directly from LP precursors (Richard et al., 2017). A shift in the pathogenicity is mainly associated with changes in or around the proteolytic HACS by substitutions, insertions or recombinations. Insertions of basic aa (Lys, Arg and rarely His) result in changing the LPAIV monobasic HACS to polybasic motifs in the HPAIV (Abdelwhab et al., 2013; Bosch et al., 1981; Richard et al., 2017).

The OIE defines two ways to determine the pathogenic potential of an AIV (Alexander, 2015; OIE, 2021a): (1) *in vivo* methods and (2) sequencing of the HACS. For one *in vivo* method, ten susceptible 6-week-old specific pathogen free or seronegative chickens are injected intravenously (IV) with allantoic fluid containing the respective virus. Chickens are examined daily for clinical signs or death and scores are given according to the severity of clinical signs from 0 (no signs) to 3 (dead) (Table 1). The average daily mean score divided by the number of observation periods (i.e., 10 days) is known as the Intravenous Pathogenicity Index (IVPI). Hence, a value of > 1.2 specifies an HP phenotype. Additionally, in the USA, high pathogenicity is determined when at least 6/8 (75%) birds die within 10 days after IV injection with an AIV.

Table 1: Scoring system for the clinical assessment of intravenously infected chickens with AIVs according to the OIE regulation for the Intravenous Pathogenicity Index (IVPI)

Score	
0	healthy chicken (no clinical signs)
1	one distinctive clinical sign
2	more than one clinical sign
3	dead chicken

Birds are considered sick or severely sick if they show one (score: 1) or more than one (score: 2) clinical signs like respiratory involvement, depression, diarrhea, cyanosis of the exposed skin or wattles, edema of the face and/or head and nervous signs (OIE, 2021a).

Although the mentioned *in vivo* methods are useful, they do not mimic the natural infection routes and are not suitable to deliver information about bird-to-bird transmission or tissue tropism of a virus. Therefore, inoculation via the ocular and/or nasal route and the pathological examination of selected birds after a defined incubation time can be carried out. Moreover, the addition of in-contact or sentinel birds indicates potential transmission events.

The second method to define an HPAIV is based on determining the aa sequence in the HACS of H5 and H7 isolates. As mentioned, HPAIVs specify polybasic cleavage sites (pCS) with several basic aa, while LPAIVs carry monobasic cleavage sites (mCS) consisting of only one basic aa. Isolates with a pCS motif identical to a previous HPAIV are also designated as highly pathogenic irrespective of the pathotyping in chickens. Isolates with novel motifs must be assessed by IVPI (OIE, 2021a).

Together, all influenza A isolates from poultry or wild birds exhibiting mortality rates of at least 75% and/or an IVPI between 1.2 and 3 or H5/H7 viruses with a polybasic HACS are HPAIVs and notifiable. Additionally, and for the purpose of the Terrestrial Code, all H5/H7 AIVs of low pathogenicity should be monitored because of their potential to mutate into a highly pathogenic phenotype. Moreover, the detection of sudden and unexpected increases in virulence of LPAIVs in poultry, as well as the detection in domestic or captive wild birds of LPAIVs that have been already naturally transmitted to humans with severe consequences, are notifiable (OIE, 2021b).

1.2.2. Natural HPAI outbreaks

The emergence of highly pathogenic avian influenza viruses is predominantly but not exclusively linked to gallinaceous poultry like chickens and turkeys (Richard et al., 2017). It is well-established that HPAIVs emerge from low pathogenic progenitors, which spread to terrestrial poultry and evolve a highly pathogenic phenotype during circulation in domestic birds for several days or even years. However, for the majority of HPAI outbreaks, no precursor virus could be identified (Richard et al., 2017) but the occurrence and spread of HPAIVs are often linked to migratory flyways of wild birds (Gale et al., 2014; Kim et al., 2021).

To date, HPAI outbreaks are restricted to H5 and H7 viruses, while fowl plague cases prior to 1959 were exclusively caused by H7 viruses (Lee et al., 2021). In 1959, the first HPAIV of subtype H5N1 was isolated in Scotland. Since then, H5 and H7 outbreaks have been documented worldwide and caused tremendous economic losses by the death or culling of infected poultry. Furthermore, the transmission of LP and HP AIVs to mammals, including humans, has been reported frequently.

1.2.3. Virulence determinants of AIVs in birds

1.2.3.1. The role of HA

The major virulence factor of AIV in birds is defined by sequence variations of the HACS and the conformation of the fusion loop, which determines the access of host proteases required for HA activation. Similar to human strains, the HACS of LPAIVs are composed of one basic aa Arg or Lys (Bosch et al., 1981; Klenk and Garten, 1994b). Those mCS are cleaved by trypsin or trypsin-like proteases in the respiratory and/or intestinal tract and lead to local infection and spread via the oral-fecal route in birds (Böttcher et al., 2006; Klenk and Garten, 1994b). In contrast, HPAIV possess a pCS with at least three basic aa and the consensus motif **R**-X-**R/K**-**R** (X is a random residue) (Abdelwhab et al., 2013; Bosch et al., 1981) where Arg at the fourth position from the carboxyl terminus of HA1 (P4) is preferential for high virulence (Kawaoka and Webster, 1988; Lee et al., 2006). The pCS is cleaved by ubiquitous furin- and subtilisin-like serine endoproteases during co-localization with the HA in the TGN (Stieneke-Grober et al., 1992). Thus, this major virulence factor can increase tissue tropism and facilitates fatal systemic infections in birds (Klenk and Garten, 1994b) (Figure 4). Although, the length of the pCS differs in naturally evolved HPAIVs, its composition and the viral background have a higher impact for pathogenicity (Abdelwhab et al., 2016b; Lee et al., 2021; Thomas, 2002). However, it was shown that recombinant viruses with five or more basic aa improved viral fitness by a preferential selection compared to viruses with fewer insertions (Luczo et al., 2018). Interestingly, also non-basic residues in or adjacent to the HACS can modulate the virulence of AIVs in poultry (Blaurock et al., 2020; Gohrbandt et al., 2011b). Apart from H5/H7 subtypes, field isolates of H9 viruses in Bangladesh with tribasic CS were shown to be processed by furin-like proteases in a recent study. Although they remained low pathogenic, an enhanced replication *in ovo* compared to other mono- or dibasic H9N2 viruses was observed (Parvin et al., 2020).

Besides the HACS motif, the presence or absence of N-linked glycans can modulate AIV virulence. While glycosylation patterns in the head domain vary among subtypes and are the main cause for shielding antigenic sites, GS in the stem domain are highly conserved and responsible for folding, trimerization and transport of HA (Klenk et al., 2002; Ohuchi et al., 1997b; Roberts et al., 1993; Tate et al., 2014). Interestingly, especially the latter were found to occasionally cover the HACS and prevent HA from proteolytic cleavage due to sterical hindrance (Kawaoka et al., 1984).

Intriguingly, it was shown that cleavage could be restored by an extended pCS motif (Kawaoka and Webster, 1989; Ohuchi et al., 1989). Moreover, potential glycosylation sites (pGS) contribute to virus replication, pH- and thermostability, chicken-to-chicken transmission and affect H5N1 virus pathogenicity in birds (Abdelwhab et al., 2016a; Klenk et al., 2002; Scholtissek, 1985; Zhang et al., 2015b).

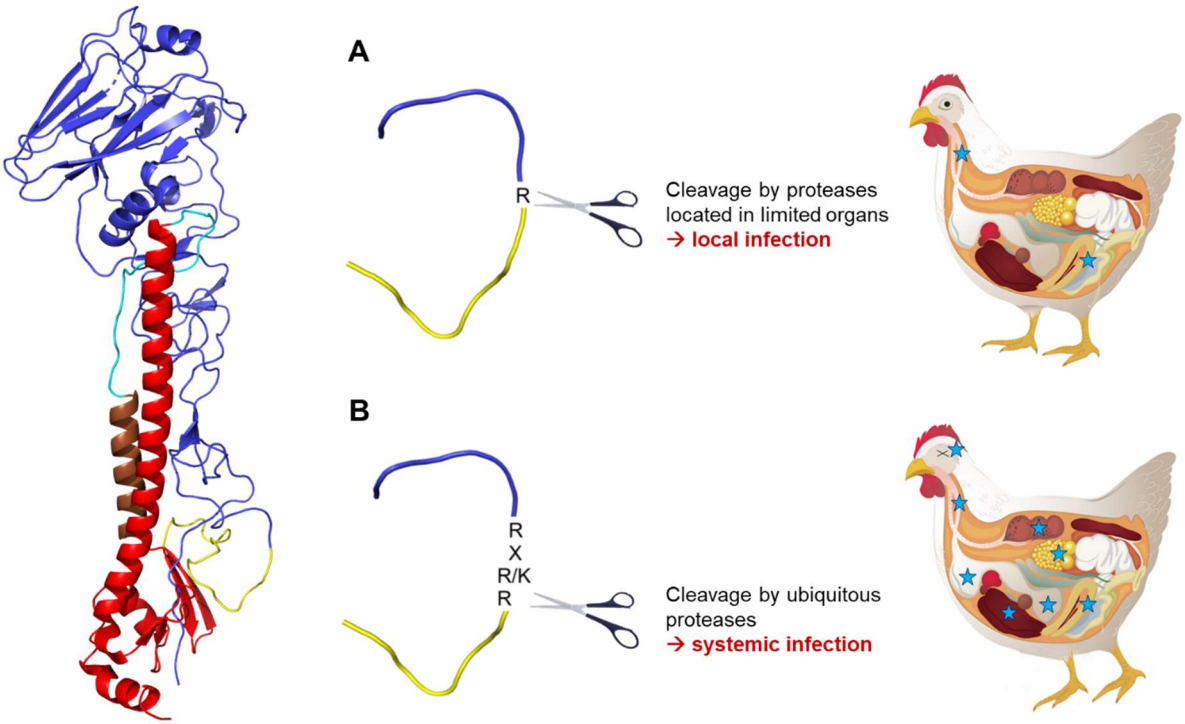

Figure 4: Determination of the pathogenicity of AIVs by the HA cleavage site. A hemagglutinin monomer with fusion loop of a (A) low pathogenic avian influenza virus with a monobasic cleavage site causes local infections in poultry or (B) highly pathogenic avian influenza virus with the consensus sequence of a polybasic cleavage site causes fatal systemic infections in poultry. Blue stars depict infiltrated organs and tissue upon infection.- *modified from Han (2020)*

1.2.3.2. The role of other gene segments

Several studies revealed that virulence determinants beyond the HA and the HACS might be necessary for highly pathogenic phenotypes in avian hosts (Bogs et al., 2010; Stech et al., 2009). The genetic comparison between LPAI progenitor viruses and HPAIVs identified several mutations in all segments. However, no specific substitution was found to serve as a prerequisite of HPAIV emergence (Richard et al., 2017). Nevertheless, studies showed that mutations in the polymerase complex and NP contributed to virulence of H5 and H7 viruses in poultry (Abdelwhab et al., 2013; Isoda et al., 2012; Tada et al., 2011; Wasilenko et al., 2008; Youk et al., 2020). Furthermore, deletions in the stalk domain of the transmembrane mushroom-shaped NA are frequently identified during adaptation of AIVs from aquatic birds to domestic poultry and were found to enhance pathogenicity, tissue tropism and chicken-to-chicken transmission (Munier et al., 2010; Stech et al., 2015). At this point, it is worth mentioning that a functional balance between HA and NA is also critical for replication and virus motility (de Vries et al., 2020; Wagner et al., 2002b). Intriguingly,

truncations or host restricted motifs at the carboxy-terminal end as well as other mutations were found to have an impact on the interferon antagonism function of NS1 and can subsequently support a highly pathogenic phenotype and increase the host range of the virus (Keiner et al., 2010; Li et al., 2006; Zielecki et al., 2010). Moreover, NS1 of contemporary H5 viruses have deletions at positions 80 – 84, which increased virulence in chickens and mice (Long et al., 2008).

1.2.4. Spread to mammalian hosts

Although the natural reservoir of AIVs was historically limited to wild birds, some avian viruses transmitted and adapted to mammalian hosts (Xu et al., 2011). Recently, two H3 IAVs of different origins became endemic in dogs in several locations globally (Harder et al., 2013; Parrish and Voorhees, 2019). To date, those lineages are limited to H1, H2, H3 and N1, N2 or N8 subtypes (Venkatesh et al., 2020) which were also found to transmit to pet animals. However, spread of AIVs from avian hosts to mammals is occasionally reported for different subtypes leading to no or limited secondary transmission. Some of these infections were fatal. By now, several avian HA subtypes have been reported to infect swine (H1-H6, H9) and marine mammals (H1, H3, H4, H5, H7, H10, H13) but were also sporadically isolated from terrestrial mammals like raccoons or minks (Kabel, 2021; Klingeborn et al., 1985; Mostafa et al., 2018; Roberts et al., 2009). Transmission of AIVs to seals, associated with pneumonic clinical signs, have been reported for H7 (Lang et al., 1981) and several non-H5/H7 (Anthony et al., 2012; Callan et al., 1995; Hinshaw et al., 1984) subtypes at the New England coast of the U.S.A. and more recently for H3N8 (Venkatesh et al., 2020), H5N8 (Kabel, 2021) and H10N7 (Zohari et al., 2014) in coastal areas of the North Sea. However, until now, no indigenous marine mammal IAV lineages have been described (Harder et al., 2013). Nevertheless, similar to pigs, seals might act as mixing vessels since the presence of both, avian-like and human-like receptors in the respiratory tract were confirmed (Anthony et al., 2012). This finding highlights a possible role of seals in the evolvement of zoonotic or even pandemic strains.

Spillover events into humans were reported for H5N1 (Subbarao et al., 1998), H5N6 (He and Duan, 2015), H6N1 (Wang et al., 2015), H7N2 (Terebuh et al., 2018), H7N3 (Tweed et al., 2004), H7N4 (Tong et al., 2018), H7N7 (Fouchier et al., 2004), H7N9 (Arima et al., 2013; Zhang et al., 2017), H9N2 (Peiris et al., 1999), H10N8 (Chen et al., 2014) and recently H10N3 (Wang et al., 2021) and H5N8 (WHO, 2021a). Human infections were acquired mainly by direct contact with infected poultry in live bird markets (LBMs) and rarely on poultry farms (Arzey et al., 2012; Chen et al., 2014; Kayali et al., 2010; Okoye et al., 2014). Importantly, human-to-human transmissions were only reported in few cases (Mostafa et al., 2018). In 1997, the first fatal cases after an infection with HPAIV H5N1 were reported in China and linked to a poultry outbreak in the Guangdong province a year before. In a second wave in 2003, this virus spread to other countries in different continents by migratory birds (Gale et al., 2014). Since then, 863 human H5N1 infections have been confirmed worldwide (as of 20-08-21), with a high case fatality rate around 53% (WHO, 2021b). Notably, human infections by AIVs are linked to subtype H9N2 or its reassortment with other AIVs.

For instance, the LPAIV and HPAIV H7N9 strains which circulate in China since 2013 and 2017, respectively, and regularly spread to humans, obtained internal genes from contemporary co-circulating H9N2 (Mostafa et al., 2018). To date, over 1568 human infections with H7N9 strains predominantly in China were confirmed (WHO, 2021b). Together, several AIV subtypes were found to spread to various mammalian hosts and frequent human infections pose a pandemic risk.

1.2.5. Virulence determinants of AIVs in mammals

1.2.5.1. The role of HA

As mentioned in paragraph 1.1.6.1, the major virulence factor for the transmission of AIVs to mammals are changes in the HA-affinity to linkage variants of the terminal Neu5Ac sialic acid of N-glycans onto host cells. A few residues in or adjacent to the HA receptor binding site can alter this binding preference from avian-like (α2,3-SA) to human-like (α2,6-SA) receptors (Byrd-Leotis et al., 2017). Many studies revealed different mutations in the RBS, which contributed to a switch in receptor affinity and increased virulence in mammals (Table S1). However, substitutions at positions 138, 155, 183, 186, 190, 226 and 228 (H3 numbering) were frequently observed in different H5/H7- and non-H5/H7 subtypes. Moreover, mutations at positions 133,135 or 158 and 160 (H3 numbering) can form glycosylation sites and were found to induce α2,6-SA binding by loss of this N-glycans in the head domain (Chang et al., 2020; Lee et al., 2018; Wang et al., 2010).

Like in avian hosts, HA stability plays a crucial role for adaptation to mammals, though with higher impact. Likewise, several studies revealed mutations predominantly in the stem region, which affect the pH of fusion and thermostability (Russier et al., 2016; Sun et al., 2019; Zaraket et al., 2013b). Thereby a decreased pH of fusion goes along with increased stability and enhanced transmissibility to and virulence in mammalian hosts due to species and cell-specific properties and adjustment of the optimal fusion time.

In contrast to avian hosts, fatal outcomes in mammals after AIV infections are rarely associated with a pCS. Individuals instead suffer from local respiratory disease symptoms like pneumonia (Karasin et al., 2000; Zohari et al., 2014) due to cleavage of mCS by tissue-restricted trypsin-like proteases, e.g., Transmembrane serinprotease 2 (TMPRSS2) and the human airway trypsin-like protease (HAT or TMPRSS11D), similar to human strains (Bottcher-Friebertshauser et al., 2010; Böttcher et al., 2006; Klenk and Garten, 1994a). Nevertheless, there is evidence that a pCS increase virus replication, transmission and pathogenicity in mammalian hosts caused by virus infiltration in tissues and organs beyond the respiratory tract (Suguitan et al., 2012; Sun et al., 2016). Interestingly, a recent study revealed an impact on HA stability and pH fusion by the tribasic motif in H9N2, which affected virulence in mice (Zhang et al., 2021).

1.2.5.2. The role of other gene segments

Beside HA, adaptation and virulence markers for mammals were identified in almost all structural and some accessory proteins (Suttie et al., 2019). Especially mutations in proteins of the RNP-complex improve polymerase activity and contribute to the increased replication and virulence in mammalian hosts (Suttie et al., 2019). Many substitutions may affect the polymerase activity, particularly PB2: E627K, D701N, A588V; PB1: N105S, K577E; PA: T97I and NP: I109T, N319K (Cheng et al., 2014; Gabriel et al., 2008; Suttie et al., 2019; Xiao et al., 2016). Intriguingly, combinations of polymerase substitutions, especially E627K of PB2, with adaptive HA mutations resulted in airborne transmitting viruses after adaptation in ferrets (Herfst et al., 2012; Sutton et al., 2014). Nevertheless, mutations in the PB1-F2, PA-X, and NP, were shown to modulate the virulence in mammalian (and avian) hosts without changes in the polymerase activity (Suttie et al., 2019). Similar to avian hosts, virulence determinants like stalk deletions of NA or C-terminal truncation of NS1 are also associated with an increased virulence in mammals. Moreover, a few substitutions in M1 and NEP are linked to enhanced viral replication in mammalian cells and increased pathogenicity (Suttie et al., 2019).

1.3. Non-H5 and H7 subtypes

1.3.1. H1-H4, H6, H8-H16

Similar to LPAI H5 and H7 viruses, the main reservoir of other AIVs are waterfowl like ducks, geese and swans, but also gulls and shorebirds. Exceptions are H13 and H16 subtypes which have a limited host range and evolved to gull-adapted lineages (Verhagen et al., 2021). Like mammalian adaptation markers, preferences of the receptor binding site exist for different bird species but are more finetuned and depend on glycan binding conformation and composition next to the terminal galactose. According to surveillance studies in mallard ducks, one of the major reservoirs of AIVs, the most prevalent HA subtypes are H4, followed by H3. H6 and H10 viruses are at the fourth and fifth place (Verhagen et al., 2021). Nevertheless, all non-H5/H7 subtypes were isolated from domestic poultry where they mainly caused, if any, mild to moderate infections with low mortality and limited spread (Mostafa et al., 2018). Intriguingly, outbreaks with high mortality rates in domestic birds caused by subtypes other than H5 or H7 have been rarely reported. In these outbreaks, co-infections with bacteria or other avian viruses like the Newcastle disease virus were described (Samy and Naguib, 2018). However, some non-H5/H7 strains naturally evolved a high virulence according to OIE regulations without such co-infections, as discussed in paragraph 1.3.2.

1.3.2. Natural shift of non-H5/H7 viruses to high virulence

Despite the frequent spread from wild birds to domestic poultry and the continuous circulation, non-H5/H7 viruses remain low pathogenic and only cause self-limiting outbreaks with mild to moderate clinical symptoms. However, there are few exceptions where non-H5/H7 expressed high lethality.

For example, in 1975, an H4N8 virus caused severe clinical symptoms in a commercial layer flock in Alabama. After consecutive passages in cell culture and chickens, a re-evaluation of this virus unveiled a highly pathogenic phenotype by killing more than 75% of intravenously infected birds and plaque formation in the absence of trypsin (Brugh, 1992). Further investigations determined that birds died from acute severe renal damage (Shalaby et al., 1994). Moreover, an H9N2 virus caused a massive drop in egg production, symptoms of HPAIVs and 10 - 30% mortality on an Indian layer poultry farm (Jakhesara et al., 2014). Another H9N2 virus with two additional, engineered basic aa in the CS evolved a highly pathogenic phenotype by killing over 75% of intravenously infected chickens after ten consecutive passages. Strikingly, the virus remained avirulent via natural routes, although it could replicate in the absence of trypsin (Soda et al., 2011). Likewise, several H10 viruses with moderate to severe virulence after intravenous infection in chickens and lethality in the field have been identified (Alexander and Spackman, 1981; Bonfante et al., 2014; Karunakaran et al., 1983; Wood et al., 1996; Zhang et al., 2016). The IVPIs of these viruses ranged from 1.27 to 1.90 (2.40 in 3-week old chicken). Like for the H4N8 virus, the deaths of birds were mainly caused by extensive replication and lesions in the kidneys (Slemons and Swayne, 1992). However, all these H10 viruses lacked a naturally evolved pCS and except for one H10N1 strain with a mortality rate of 47.6%, no non-H5/H7 virus caused lethality after inoculation via natural routes (Table 2).

1.3.3. High virulence of recombinant non-H5/H7 viruses

Besides naturally evolved viruses with high virulence, a few studies investigated recombinant viruses with engineered pCS in non-H5/H7 viruses (Table 2). Accordingly, Veits et al. (2012) investigated the virulence of different non-H5/H7 subtypes after the insertion of a pCS mimicking the cleavage site of an HPAIV H5N2 virus and gene segments from an HPAIV H5N1 virus. Interestingly, the modified HA-subtypes H2, H4, H6, H8 and H14 showed a highly virulent phenotype after oculonasal infections in chickens. Strikingly, the reassortants of subtype H2, H4, H8 and H14, which showed a mortality rate of 100% via the natural route, revealed IVPIs between 2.30 and 2.85 comparable to HPAIV H5 or H7 strains. Moreover, Gohrbandt et al. (2011) followed a similar approach for the H9 subtype and revealed high pathogenicity with an IVPI of 1.23 after reassortment with HPAIV H5N1 and insertion of its pCS (Gohrbandt et al., 2011a). However, both studies determined no shifts to high virulence when engineered HAs were expressed in the background of LPAIVs. In contrast, Munster et al. (2010) investigated an H6N1 virus with a synthetic pCS similar to an HPAIV H5N1. Surprisingly, the recombinant virus exhibited systemic spread upon intranasal inoculation and an IVPI of 1.40 in the parental virus background. This indicates the possibility of non-H5/H7 viruses to shift to high virulence via natural routes in the appropriate viral background.

Table 2: Recombinant and naturally evolved non-H5/H7 AIVs with high virulence in chicken

Subtype	Gain of function	HACS*	Viral Background / Reassortment	Pathogenicity index (PI)	Mortality**	IVPI	Reference
H2N5	recombinant	PQ**RRRKKR**/G	A/Swan/Germany/R65/2006 (H5N1)	2.23	100%	2.79	(Veits et al., 2012)
H4N6	recombinant	PQ**RRRKKR**/G	A/Swan/Germany/R65/2006 (H5N1)	2.05	100%	2.37	(Veits et al., 2012)
H4N8	naturally evolved / consecutive passages	IPE**K**AT**R**/G	A/chicken/Alabama/7395/75 (H4N8)	-	75% (IV)	-	(Brugh, 1992; Shalaby et al., 1994; Slemons and Swayne, 1992)
H6N2	recombinant	PQ**RRRKKR**/G	A/Swan/Germany/R65/2006 (H5N1)	1.58	75%	-	(Veits et al., 2012)
H6N1	recombinant	ET**RRRKKR**/G	A/Mallard/Sweden/81/02 (H6N1)	-	0%	1.41	(Munster et al., 2010)
H8N4	recombinant	PQ**RRRKKR**/G	A/Swan/Germany/R65/2006 (H5N1)	2.40	100%	2.85	(Veits et al., 2012)
H9N1	recombinant	S**RRRRKKR**/G	A/Swan/Germany/R65/2006 (H5N1)	2.35	100%	1.23	(Gohrbandt et al., 2011a)
H9N2	naturally evolved	PA**R**SS**R**/G	A/chicken/UP/India/2543/2004 (H9N2)	-	10 – 30%	-	(Jakhesara et al., 2014)
H9N2	engineered HACS / consecutive passages	PA**RKKR**/G	A/chicken/Yokohama/aq-55/2001 (H9N2)	-	75%	-	(Soda et al., 2011)
H10N1	naturally evolved	PEIMQG**R**/G	A/mallard/Italy/4518/2007 (H10N1)	-	47.6%	1.90 – 2.40	(Bonfante et al., 2014)
H10N3	naturally evolved	PEIMQG**R**/G	A/duck/Fujian/1761/2010 (H10N3)	0.00	0%	1.60	(Zhang et al., 2016)

H10N4	naturally evolved	PEIMQG**R**/G	A/turkey/England/384/79 (H10N4)	-	70% (IV)	1.34 - 1.62	(Alexander and Spackman, 1981; Wood et al., 1996)
H10N5	naturally evolved	PEIMQG**R**/G	A/mandarin duck/Singapore/805/F-72/7/93 (H10N5)	0.00	0% 80% (IV)	1.56 – 1.76	(Wood et al., 1996)
H10N7	naturally evolved	PEVVQG**R**/G	A/turkey/Minnesota/5/1979 (H10N7)	-	31%	-	(Karunakaran et al., 1983)
H10N7	naturally evolved	PEIMQG**R**/G	A/duck/Shanxi/3180/2010 (H10N7)	0.00	0%	1.27	(Zhang et al., 2016)
H10N7	naturally evolved	PEIMQG**R**/G	A/duck/Italy/1398/2006 (H10N7)	-	-	>1.20	(Bonfante et al., 2014)
H14N3	recombinant	PQ**RRRKKR**/G	A/Swan/Germany/R65/2006 (H5N1)	1.63	100%	2.61	(Veits et al., 2012)

* Hemagglutinin cleavage site of indicated viruses: basic amino acids Arginine (R) and Lysine (K) are shown in bold letters; underlined motifs mark engineered amino acid insertions

** Mortality after natural infection (e.g., oculonasal or intranasal), mortality upon intravenous infection are indicated with (IV).

1.3.4. H4Nx viruses in birds and mammals

H4 is one of the most common HA subtypes and belongs to the LPAIVs. Since the first isolation in 1956 from a duck in former Czechoslovakia (Song et al., 2017), those viruses have been reported to be widely circulating in Asia, Europe and North America. H4 viruses showed a broad host range in wild and domestic avian species as well as in mammals (Brugh, 1992; Hinshaw et al., 1984; Hu et al., 2017; Karasin et al., 2000; Okuya et al., 2017; Reid et al., 2018; Roberts et al., 2009). Because of the low pathogenic character, outbreaks of H4Nx viruses often remain unnoticed or are self-limiting, and their isolation depends on comprehensive surveillance studies (Hollander et al., 2019; Kang et al., 2013).

Several studies revealed a high prevalence of H4 infections, particularly in LBMs in Asia, North American and European countries (Teng et al., 2012; Wisedchanwet et al., 2011; Xu et al., 2014). Also, reassortments of H4 viruses with other AIV subtypes have been frequently reported (Bui et al., 2012; Teng et al., 2012; Wu et al., 2014; Xie et al., 2017; Yuan et al., 2015). Transmissions to domestic birds are often associated with the migratory flyway of wild birds, while some studies showed that contaminated water might play a role (Hollander et al., 2019; Hu et al., 2017; Ornelas-Eusebio et al., 2015; VanDalen et al., 2010). Despite the general low pathogenicity of H4Nx viruses, they rarely cause disease or even mortality in birds under field conditions, as discussed in paragraph 1.3.2 (Brugh, 1992; Yuan et al., 2015).

Furthermore, a few avian H4 isolates were able to transmit and replicate in mice and guinea pigs without prior adaptation and even led to significant body weight losses and severe respiratory diseases with the eventual death of some mice (Bui et al., 2012; Hu et al., 2017; Kang et al., 2013). Interestingly, passaging of an avian H4N6 virus in mice led to aa exchanges in PB2 and HA, enhancing polymerase activity and/or increased virulence (Xu et al., 2019).

Naturally occurring interspecies transmissions of avian H4 viruses to mammals have been reported for seals (Gulyaeva et al., 2018; Hinshaw et al., 1984), raccoons (Roberts et al., 2009) and pigs (Abente et al., 2017; Karasin et al., 2000; Ninomiya et al., 2002) with low to fatal respiratory diseases. Notably, in one study, the sequence comparison of two H4N6 viruses revealed two critical aa substitutions (Q226L, G228S) in the receptor binding domain of the HA responsible for human-like receptor preference of the swine isolate (Song et al., 2017). This indicates the potential of H4Nx to cross species barriers and possibly adapt and spread to humans. Nevertheless, to date, no H4 virus was isolated from humans. However, serological studies of adults regularly exposed to poultries indicated previous infections with H4 viruses (Kayali et al., 2011; Kayali et al., 2010).

1.3.4.1. A/Quail/California/D113023808/2012 (H4N2)

In August 2012, an AIV from subtype H4N2 designated A/Quail/California/D113023808/2012 (hereafter QU/CA12) was isolated from farmed quails in California with a mortality rate of less than 1.6%. Sequence and phylogenetic analyses revealed that the genes coding for both surface glycoproteins closely matched with a low pathogenic H4N2 strain from Peking duck isolated already six years earlier in the same U.S. state. Strikingly, the unique feature of the quail virus was the acquisition of a pCS, the major virulence marker usually seen in H5 and H7 AIV strains only. Compared to the LP H4 precursor carrying the monobasic CS motif PE**K**TT**R**/G, QU/CA12 exhibited the polybasic motif PE**KRR**T**R**/G. However, virus replication in avian and mammal cell cultures remained trypsin-dependent. No morbidity or mortality was observed after intranasal or intravenous infection of experimental chickens, indicating a typical LP phenotype (Wong et al., 2014).

1.3.5. H10Nx viruses in birds and mammals

The first-ever isolated LPAIV was of subtype H10N7 and derived from a chicken in Germany. This so-called “Dinter virus” was isolated in 1949 (Lee et al., 2021). Since then, H10 viruses have been isolated from wild birds and poultry worldwide and were associated with all possible NA N1-N9 subtypes (Wille et al., 2018). Surveillances in LBMs in China identified many H10 viruses and determined the risk of reassortment with other strains and subtypes (Deng et al., 2015; Hu et al., 2015; Ma et al., 2015; Wu et al., 2015a). In poultry, H10 viruses are generally LP with few exceptions (see above) (Abolnik et al., 2010; Alexander and Spackman, 1981; Bonfante et al., 2014; Karunakaran et al., 1983; Wood et al., 1996; Zhang et al., 2016). There are several reports on the potential of H10Nx viruses to spread to mammals. In 1984, an H10N4 virus was isolated from a mink in Sweden. In contrast to the chicken isolate from 1949, this virus had the ability for multiple-cycle replication in mink airways with a critical role for the different NS1 to evade the initial host immune response (Englund, 2000; Klingeborn et al., 1985; Zohari et al., 2010). Furthermore, several H10 viruses were detected in pigs, dogs and raccoons (Hall et al., 2008; Su et al., 2014; Wang et al., 2012). Notably, in spring and summer 2014, infections with an H10N7 virus were reported after mass death of harbor and grey seals due to severe pneumonia upon bacterial co-infection in northern Europe (Bodewes et al., 2015; van den Brand et al., 2016; Zohari et al., 2014).

Under laboratory conditions, several H10Nx viruses caused moderate to severe pathogenicity in mice (El-Shesheny et al., 2018; Qi et al., 2014; Wu et al., 2015a, b). Genome sequencing of mammal-adapted H10 viruses unveiled several adaptation markers like PB2 E627K, PA T97I and HA G409E (Wu et al., 2016; Zhang et al., 2016). Experimental investigations of mammalian and bird isolates revealed specificities for avian α2,3 receptors by H10 viruses, but some strains exhibited a dual receptor binding specificity with a preference for α2,6-SA and showed an elevated NA activity similar to human IAVs (Sutton et al., 2017; Vachieri et al., 2014; Wu et al., 2016; Yang et al., 2015a). A recent study with TMPRSS2 deficient mice determined a critical role for this

protease regarding the proteolytic activation of H10 viruses in mammals (Lambertz et al., 2019). In late 2013 and early 2014, the first human cases with a fatal outcome after infection with an H10N8 virus in South China were reported (Chen et al., 2014; Ma et al., 2015). All segments derived from an avian origin H10 after a multiple reassortment with an HxN8 virus and six internal genes of H9N2. Notably, H10N8 maintained an avian-like receptor binding preference. However, mammalian adaptation markers in HA, M1, NS1 and PB2 could be determined (Chen et al., 2014; To et al., 2014). Recently, another human infection caused by an H10N3 virus was confirmed. Initial sequencing data revealed the G228S mutation in the HA, indicating a preference for human-like receptors (Wang et al., 2021). This suggests that H10 viruses are able to cross species barriers and can lead to fatal infections in mammals and humans.

2. Objectives

Avian influenza viruses infect a wide range of host species and exhibit low or high pathogenicity phenotypes in birds and mammals. In poultry, natural HPAIVs are predominantly restricted to H5 or H7 subtypes with a pCS in the hemagglutinin that can be cleaved by ubiquitous furin- and subtilisin-like proteases and thus facilitates systemic infections with significant economic and ecologic losses in terrestrial and aquatic birds. Therefore, HPAIVs are notifiable to the OIE and infected flocks should be culled to control the spread of the virus. Conversely, non-H5/H7 viruses are frequently isolated from wild birds and poultry without causing apparent clinical signs, though losses in poultry flocks were also reported after the infection with, e.g., H3, H4, H6 and H9 viruses. Therefore, non-H5/H7 viruses are not notifiable to the OIE and no countermeasures have to be implemented to eradicate the infected flocks. Consequently, many non-H5/H7 AIVs are endemic in poultry worldwide. To date, non-H5/H7 viruses did not evolve to HP in nature and mostly retained the mCS. The H4N2 virus isolated from quails in California in 2012 represents one of the few exceptions for non-H5/H7 due to the presence of a natural pCS. Several laboratory experiments showed the ability of some non-H5/H7 viruses to shift to high virulence after the acquisition of synthetic polybasic HACS and gene segments from an HPAIV H5N1. Therefore, there was a need to assess the risk for the transition of this unique H4N2 to an HPAIV.

Moreover, beside H5 and H7 strains, non-H5/H7 AIVs are transmitted frequently from wild or domestic birds to mammals, including humans and pose a potential pandemic risk. Although several key mutations are linked to mammal adaptation, many studies were based on H5, H7 or H9 and less is known about adaptation markers of other non-H5/H7 AIVs in mammals. In 2014 an H10N7 virus caused mass death in harbor seals in northern Europe and its HA showed 98 – 99 % identity to an H10N4 virus from mallards. Nevertheless, compared to AIVs of avian-origin, the seal virus possessed unique mutations in the HA1 domain, including the receptor binding site and HACS. The impact of these mutations on virus replication, fitness and receptor binding affinity can give new insights into the adaptation of non-H5/H7 AIVs to mammalian hosts.

In this dissertation, genetic determinants for high virulence and adaptation of H4N2 and H10N7 viruses in birds and mammals respectively were studied. In the first and second publication, the impact of mutations in or adjacent to the HACS with or without the reassortment with HPAIVs H5/H7 on the virulence of the unique H4N2 virus in chickens was assessed. The third publication studied the biological impact of HA1 mutations on the potential adaptation of seal H10N7 viruses in mammals. Using reverse genetics, H4N2 and H10N4 with specified mutations or genetic constellations were rescued. The recombinant viruses were characterized *in vitro* and/or in *vivo*.

3. Publications

I

(I) The role of glycosylation in the N-terminus of the hemagglutinin of a unique H4N2 with a natural polybasic cleavage site in virus fitness *in vitro* and *in vivo*

Marcel Gischke, Ola Bagato, Angele Breithaupt, David Scheibner, Claudia Blaurock, Melina Vallbracht, Axel Karger, Beate Crossley, Jutta Veits, Eva Böttcher-Friebertshäuser, Thomas C. Mettenleiter and Elsayed M. Abdelwhab

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The role of glycosylation in the N-terminus of the hemagglutinin of a unique H4N2 with a natural polybasic cleavage site in virus fitness *in vitro* and *in vivo*

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ABSTRACT

To date, only low pathogenic (LP) H5 and H7 avian influenza viruses (AIV) have been observed to naturally shift to a highly pathogenic (HP) phenotype after mutation of the monobasic hemagglutinin (HA) cleavage site (HACS) to polybasic motifs. The LPAIV monobasic HACS is activated by tissue-restricted trypsin-like enzymes, while the HPAIV polybasic HACS is activated by ubiquitous furin-like enzymes. However, glycosylation near the HACS can affect proteolytic activation and reduced virulence of some HPAIV in chickens. In 2012, a unique H4N2 virus with a polybasic HACS was isolated from quails but was LP in chickens. Whether glycosylation sites (GS) near the HACS hinder the evolution of HPAIV H4N2 remains unclear. Here, we analyzed the prevalence of potential GS in the N-terminus of HA1, ^{2}NYT^{4} and ^{18}NGT^{20}, in all AIV sequences and studied their impact on H4N2 virus fitness. Although the two motifs are conserved, some non-H5/H7 subtypes lack one or both GS. Both sites were glycosylated in this H4N2 virus. Deglycosylation increased trypsin-independent replication in cell culture, cell-to-cell spread and syncytium formation at low-acidic pH, but negatively affected the thermostability and receptor-binding affinity. Alteration of ^{2}NYT^{4} with or without ^{18}NGT^{20} enabled systemic spread of the virus to different organs including the brain of chicken embryos. However, all intranasally inoculated chickens did not show clinical signs. Together, although the conserved GS near the HACS are important for HA stability and receptor binding, deglycosylation increased the H4N2 HA-activation, replication and tissue tropism suggesting a potential role for virus adaptation in poultry.

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Introduction

Avian influenza viruses (AIV) belong to the genus Influenza A Virus in the family *Orthomyxoviridae*. The genome of AIV consists of eight segments of single-stranded RNA of negative polarity [1,2] which encode more than 10 viral proteins [1]. AIV are classified according to the antigenic variants of the two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), into 16 HA (H1 – H16) and 9 NA (N1 – N9) subtypes with 144 possible HxNy combinations. All HA and NA subtypes were isolated from wild birds, the natural reservoir of the virus [3]. Low pathogenic (LP) AIV of subtypes H5 and H7 can spontaneously mutate into highly pathogenic (HP) viruses. While most AIV subtypes are low pathogenic in the natural reservoir, transition to HP viruses mostly occurs during circulation in domestic poultry [4,5].

Although the virulence of H5 and H7 viruses is polygenically determined, mutations in the HA cleavage site (HACS) are the main determinant of virulence in poultry. Mutation of the monobasic HACS in LPAIV to a polybasic HACS in HPAIV is a prerequisite for high virulence and replication. The HA of LPAIV is activated by trypsin or trypsin-like enzymes (e.g. the human airway trypsin-like protease HAT or the transmembrane protease serine 2 TMPRSS2) in the gastrointestinal or respiratory tracts of birds. Thus, these viruses cause localized infections and mild, if any, clinical signs. Conversely, HPAIV are activated by furin-like enzymes ubiquitously expressed in all tissues causing systemic infection and death [6–8]. Intriguingly, some H5 viruses with polybasic HACS motifs exhibited low virulence in chickens [5,9]. N-Glycosylation, i.e. carbohydrate side chains attached to asparagine

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Supplemental data for this article can be accessed here.

residues, of the HA of HPAIV plays diverse roles in protein stability, receptor binding affinity, fusion activity, immune-evasion, virulence and bird-to-bird transmission [10–15]. The N-terminal glycosylated residues near the HACS are highly conserved among all AIV subtypes [16]. The carbohydrate side chain linked to the asparagine (N) can sterically hinder the access of proteases to the HACS loop-like structure and, thus, modulate virulence by limiting access of furin-like proteases to the HACS [10,17]. A well-known natural example is the A/chicken/Pennsylvania/1983(H5N2) virus, which possessed a polybasic HACS but was avirulent in chickens. Removal of glycosylation by a mutation of asparagine in position 11 in the HA1 or increasing the number of basic amino acids (aa) in the HACS enabled trypsin-independent HA activation and a shift to high pathogenicity [10]. Conversely, removal of the same glycosylation site (GS) in a HPAIV A/Mallard/Huadong/S/2005(H5N1) revealed a significantly delayed HA cleavability and a decreased virus fitness *in vivo* and *in vitro*, while the experimentally modified GS reverted after few virus passages [18].

Some non-H5/H7 viruses exhibited high virulence after acquisition of a polybasic CS with or without reassortment with gene segments from HPAIV H5N1 [19–21]. However, it is unknown why HPAIV are restricted in nature to AIV of H5 and H7 subtypes. In each HPAIV H5/H7, acquisition of a specific polybasic HACS accompanied the shift to high virulence [5]. In 2012, an H4N2 virus was isolated from farmed quails in California with a history of 1.6% mortality [22]. Sequence analysis revealed that the HA possessed a polybasic HACS motif ^{322}PEKRRTR/G^{329} [22]. This is the only known non-H5/H7 virus with 4 basic aa in the HACS, which fulfills the criteria for the HPAIV furin-specific motif (R-X-K/R-R) [23]. Nevertheless, the virus exhibited a LP phenotype in chickens without causing morbidity or mortality, and did not grow on MDCK cells without trypsin [22]. The HA possesses five potential N-linked glycosylation sites (pGS) ^{2}NYT^{4}, ^{18}NGT^{20}, ^{129}NGT^{131}, ^{162}NLT^{164} and ^{294}NVS^{296} in the HA1 subunit and ^{482}NGT^{484} in the HA2 subunit [22], where ^{2}NYT^{4} and ^{18}NGT^{20} are located in the HA1 N-terminus close to the HACS region. It is unknown whether glycosylation near the HACS can affect the virulence of this unique H4N2 virus in chickens.

In this study, we analyzed pGS at positions 2 and 18 in all HA sequences of the HxNy AIV available in the Global Initiative on Sharing Avian Influenza Data (GISAID) and GenBank to 27 January 2020. Using reverse genetics, the wild-type virus (designated rgH4N2) and three recombinant H4N2 viruses with substitutions in position 2 (designated rgN2S), at position 18 (rgN18D) or at both sites (rgN2S/N18D) were rescued. The impact of the N2S and/or N18D mutations on virus fitness *in vitro* and in chickens *in vivo* was studied.

Materials and methods

Ethics statement

The animal experiment in this study was carried out after approval by the authorized ethics committee of the State Office of Agriculture, Food Safety and Fishery in Mecklenburg – Western Pomerania (LALLF M-V permission number 7221.3–1.1-051-12) and according to the German Regulations for Animal Welfare. The commissioner for animal welfare at the FLI representing the Institutional Animal Care and Use Committee (IACUC) approved the experiment, which was conducted in the BSL3 laboratory and animal facilities of Friedrich-Loeffler-Institut (FLI), Insel Riems, Germany.

Sequence analysis

Sequences of the HA of AIV were retrieved from GISAID and the Influenza Virus Database of the National Centre for Biotechnology Information (NCBI). After removal of identical and laboratory-viruses, sequences were aligned using Multiple Alignment using Fast Fourier Transform (MAFFT) [24]. pGS was predicted by the sequon motif N-X-S/T, where X can be any amino acid (aa) except proline. The location of indicated aa on the tertiary structure of the HA was imposed using deduced aa sequence of HA of H4N2 virus using SWISS MODEL (http://swissmodel.expasy.org/) and then viewed in Geneious Prime (Biomatters, Version 2019.2.3) and edited manually. Numbering of aa in this study is based on the mature H4 protein after removal of the signal peptide.

Viruses, plasmids and cells

A/quail/California/D113023808/2012(H4N2) was obtained from California Animal Health and Food Safety Laboratory System, Department of Medicine and Epidemiology, University of California, Davis. A/Victoria/1975(H3N2) was kindly provided by S. Pleschka and A. Mostafa from Justus-Liebig-University, Gießen, Germany. A/swan/Germany/R65/2006(H5N1) was obtained from the repository of the FLI kindly provided by Timm C. Harder. pHWS-plasmids containing all gene segments from this H4N2 virus were successfully cloned in a previous

study [25]. pCAGGS plasmids encoding HAT and TMPRSS2 have been described previously [26]. pCAGGS plasmid was kindly provided by Stefan Finke, FLI. Plasmid expressing the GFP (pEGFP-N1, Clontech) under control of the human cytomegalovirus immediate-early 1 promoter/enhancer was kindly provided by Barbara Klupp, FLI. MDCK-HAT and MDCK-TMPRSS2 cells, which express HAT and TMPRSS2, respectively were obtained from Marburg University, and other cell lines used in this study were obtained from the Cell Culture Collection in Veterinary Medicine of the FLI. Primary chicken embryo kidney (CEK) cells were prepared from 18-day-old embryonated chicken eggs (ECE) according to the standard procedures [27].

Generation of recombinant viruses and mutagenesis

The pHWS-HA plasmid of A/quail/California/D113023808/2012(H4N2) was used for mutagenesis using QuikChange II Site-Directed Mutagenesis Kit (Agilent, Catalog #200,523). Changing ^{2}NYT^{4} and ^{18}NGT^{20} to ^{2}SYT^{4} and ^{18}DGT^{20} to generate N2S and N18D as found naturally in some AIV, respectively, was successfully done using the following primers: N2S_F: 5′-GGGATACTCTCAGAGCTACACAGG AAATCCTGTG-′3 and N2S_R: 5′-CACAGGATTT CCTGTGTAGCTCTGAGAGTATCCC-′3 for deglycosylation at position 2 and N18S_F: 5′ CGTCATGCCGTATC TGATGGAACAATGGTAAAAACC-′3 and N18S_R: 5′-GGTTTTTACCATTGTTCCATCAGATACGGCATGAC-G-′3 for deglycosylation at position 18. Three HA plasmids carrying N2S, N18D or both N2S and N18D were successfully generated. Furthermore, the HA gene and mutated variants of H4N2 as indicated below were cloned from pHWS-HA into pCAGGS expression vector by conventional restriction digest and ligation. Four recombinant viruses carrying the HA from wild-type H4N2 (designated rgH4N2), N2S (designated rgN2S), N18D (designated rgN18D) or both mutations (rgN2S/N18D) and the other gene segments from rgH4N2 were rescued by transfection of MDCKII/HEK293T co-culture as previously described [28]. Two days post transfection supernatant was inoculated into 9-to-11 day-old specific pathogen free (SPF) embryonated chicken eggs (ECE) (VALO BioMedia GmbH) as recommended [29]. Allantoic fluid (AF) with a titer >16 ($4\log_2$) was tested for bacterial contamination on Columbia sheep blood agar. Virus stocks were stored at −70°C until use. To exclude unwanted mutations, viral RNA was extracted from virus stocks using the QIAamp Viral RNA Mini Kit and transcribed into cDNA using the Omniscript RT Kit (Qiagen, Catalog #205,111) and segments were amplified by Phusion RT-PCR kit [28]. Gel slices were extracted using Qiagen Gel Extraction kit (Qiagen, Catalog #28,706) and subjected to Sanger sequencing using ABI BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems, Catalog #4,337,452). Plaque forming units (pfu) of virus stocks were determined as described below.

Multicycle replication kinetics

Growth kinetics were performed in CEK in 12-well plates at a multiplicity of infection (MOI) of 0.001 pfu for 8 and 24 h post-infection (hpi). Infection experiments of MDCK, MDCK-HAT and MDCK-TMPRSS2 cells were performed for 24 h only. Briefly, cells were infected for 1 h at 37°C and 5% CO2, incubated for 2 min with citric acid buffer (pH 3.0), then washed twice with 1x phosphate-buffered saline (PBS) and covered with Minimum Essential Medium (MEM) containing 0.2% bovine serum albumin (BSA) (MP Biomedicals, Catalog #9048-46-8) with antibiotics (penicillin-streptomycin) in the presence or absence of 2 µg/µl of N-tosyl-L-phenyalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma Aldrich, Catalog #4,370,285). At the indicated time points, cells and supernatants were collected and stored at −70°C until used for virus titration by plaque assay as described below. All experiments were conducted in three independent replicates. Results are expressed as average and standard deviation for all replicates.

Virus titration

Virus titration was performed by plaque assay. Briefly, confluent MDCKII cells in 12-well plates were incubated with 10-fold dilutions of recombinant viruses at 37°C and 5% CO_2. After an hour, cells were washed twice with 1 x PBS and covered by semi-solid Bacto^{TM} Agar (BD, Catalog #90,000-767) with 50% MEM containing 4% BSA and 1 µg/µl trypsin without antibiotics. The plates were incubated for 3 days at 37°C and 5% CO_2, and then fixed for 48 hours using 10% formaldehyde containing 0.1% crystal violet. After removal of the Bacto^{TM} Agar, plates were rinsed with distilled water, left to dry and the plaques were counted. Viral titers were expressed as plaque-forming units per ml (pfu/ml).

Cell-to-cell spread

To study the impact of deglycosylation on cell-to-cell spread with and without trypsin, MDCKII cells were infected with recombinant viruses for 1 h and further processed as described above using plaque assay. After fixation with 10% formaldehyde containing 0.1% crystal

violet, the diameter of 50 plaques was measured by microscopy using Eclipse Ti-S with software NIS-Elements, version 4.0; Nikon. Diameter of plaques of the rgH4N2 in the absence of trypsin was adjusted to 100%. The plaque size obtained by different recombinant viruses was calculated relative to the rgH4N2. Results are expressed as relative mean and standard deviation.

Western blot

Glycosylation of the HA was confirmed using N-Glycosidase F (PNGase F, New England BioLabs, Catalog #P0704S) in standard Western Blot procedures with few modifications [30]. Briefly, cells in 12-well plates were infected at MOI of 1 for one hour. Cells were washed with PBS and MEM containing BSA with trypsin was added to each well. Infected MDCK cells were incubated for 8 h at 37°C and then collected in 2 ml Eppendorf tubes. Cells were washed twice with 1x PBS followed by centrifugation at 14,000 rpm for 15 min. Enzymatic deglycosylation by N-Glycosidase F (PNGase F, New England BioLabs, Catalog #P0704S) of N2 and N18 was done according to the producer's guidelines. Proteins were denatured in Laemmli buffer (Serva, Catalog #42,556.01) containing 5% 2-mercaptoethanol for 5 min at 99°C. Cell lysates along with BenchMark™ Pre-Stained Protein Ladder marker were separated by discontinuous sodium dodecyl sulfate and 10% polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes using a blotting device at 20 V for 90 min and membranes were blocked for 1 h in 5% skim milk solution. The HA protein was detected with AIV polyclonal anti-HA H4N2 antibodies derived from rabbits immunized with an H4N2 HA2 specific-peptide. The blots were incubated with primary antibodies overnight followed by washing with TBS buffer with 0.25% Tween®20. Secondary peroxidase-conjugated anti-rabbit IgG for HA (Jackson Immuno Research, Catalog #111-035-144) were used at a dilution 1:20,000. The immunodetection was done by chemiluminescence using Clarity™ Western ECL Substrate (BioRad, Catalog #1,705,061). Images were acquired by a Bio-Rad Versadoc 4000 Molecular Imager (BioRad, Catalog #170-8640) and analyzed by Quantity One software (BioRad).

Receptor-binding assay

Affinity to the avian α2,3-linked sialic acid (SA) receptors was determined by a solid-phase binding assay [31,32]. Briefly, viruses were adjusted to 10^5 pfu/ml or 32 HA units. Plates pre-coated with 10 µg/ml fetuin (Sigma Aldrich, Catalog #F3004) were incubated with 50 µl viruses overnight at 4°C. Unbound virus was aspirated and the plates were washed with 2x PBS and blocked overnight at 4°C by 0.1% neuraminidase inactivated BSA. Afterward the plates were washed with 2x PBS + Tween® 80 (0.01%) and incubated with a 1:2 serial dilution of HRP-fetuin containing α2,3-SA for 1 h at 4°C. The plates were washed again and incubated for 30 min at room temperature (rt) in the dark. The reaction was stopped with 50 µL of 3% H_2SO_4 and the optical density (OD) was measured at 450 nm using Tecan ELISA Reader.

Fusion assay

The effect of glycosylation at positions 2 and 18 on pH-dependent fusion activation of the recombinant viruses was studied as previously described [33,34], with few modifications. Briefly, QM-9 cells in 24-well plates were transfected using Lipofectamine2000® with 0.6 µg pCAGGS-plasmid containing HA from rgH4N2, N2S, N18D and N2S/N18D and GFP plasmid (100 ng/µl) to facilitate the evaluation of the assay. Transfected cells were covered with MEM containing 5% FCS, and incubated for 16 hours at 37°C and 5% CO_2. Thereafter, supernatant was removed and the cells were treated with MEM containing BSA and 0.05% trypsin for 10 min at rt for proteolytic activation of HA. Furthermore, the cells were incubated for 15 min with MEM containing 10% FCS. Medium was removed and PBS fusion buffers previously adjusted to pH 4.0, 4.2, 4.4, 4.6, 4.8, 5.0, 5.2, 5.4, 5.6, 5.8 and 6.0 were added for 4 min. The cells were then washed with 1x PBS, and MEM with 10% FCS was added. After 4 hours incubation at 37°C, 4% paraformaldehyde (PFA) was added for 10 min and cells were washed with PBS. Syncytia formation was measured by fluorescence microscopy (Eclipse Ti-S with software NIS-Elements, version 4.0; Nikon) and the average area of syncytia was calculated. The pH threshold was the highest pH value at which fusion was observed.

Heat stability

Viruses were adjusted to 10^5 pfu/ml or 32 HA units and incubated at 50°C for 0, 0.5, 1, 2, 4, and 6 hours in duplicates and then stored at −70°C for titration. HA test and plaque assay were used to determine the HA activity and infectivity, respectively, as previously described [29]. The HA test and plaque assay were conducted in duplicates for each virus. The results are shown as relative average and standard deviation of all experiments for each virus.

Histopathology and immunohistochemistry

Four 14-day-old SPF ECE were inoculated with 10^4 pfu of each virus via the allantoic sac. Eggs were candled daily for 4 days and subsequently collected in 10% neutral buffered formalin. Tissue samples were paraffin-embedded and 2-3-µm-thick sections were stained with hematoxylin and eosin (HE). The severity of necrotizing inflammation was scored on an ordinal 0 to 3 scale: 0 = no change; 1 = mild; 2 = moderate, and 3 = severe necrosis. The distribution of recombinant viruses in different organs and tissues were studied by immunohistochemistry as described [35] using a primary antibody against the M1 protein of IAV (ATCC clone HB-64). The extent of viral antigen labeling was scored on a 0 to 3 scoring scale: 0 = no antigen, 1 = focal to oligofocal, 2 = multifocal, 3 = coalescing/diffuse.

Chicken experiment

SPF ECE from white leghorn chickens were purchased from VALO BioMedia GmbH. Eggs were incubated at the experimental animal facilities of the FLI until hatch. Six-week-old male and female chickens were randomly allocated into four groups. Five birds were inoculated via the oculonasal (ON) route with 10^5 pfu of each virus on both sides (~100 µL in each side). Clinical signs were monitored daily and mortality for 10 dpi. Oropharyngeal (OP) and cloacal (CL) swabs were collected at 2, 4, 7 and 10 dpi using MEM. Viral RNA was extracted from swab media using Nucleo Mag® VET Kit (Macherey & Nagel, Catalog #744,200.4) according to the manufacturer's instructions using the KingFisher Flex Purification System (Thermo Fisher Scientific, Catalog #5,400,630). The relative amount of viral load in the swabs was determined by generic real-time-reverse-transcription polymerase chain reaction (RT-qPCR) targeting the AIV Matrix gene [36]. Standard curves were generated by serial dilutions of H4N2 virus in each RT-qPCR plate. Quantification was performed by plotting the CT-value of a given sample against the known viral dilution in the standard curves. Results are expressed as equivalent log10 pfu/ml as arithmetic mean with standard deviation. At the end of the experiment, all surviving birds were euthanized after Isoflurane® (CP-Pharma, Catalog #1214) inhalation. Blood was collected and sera were tested for anti-AIV NP antibodies using ID screen Influenza Antibody Competition Multispecies kit (IDvet, Catalog #FLUACA-2P) following the instructions of the manufacturer.

Statistics

Statistical analyses were done by GraphPad Prism 8 software (CA, Version 8.4.3). Differences in replication kinetics, receptor-binding activity, size of syncytia and heat stability experiments were analyzed using ordinary one-way ANOVA with post hoc Tukey tests. Plaque size and RT-qPCR results were evaluated using ordinary one-way ANOVA with Bonferroni correction. A p-value < 0.05 was considered significant.

Results

pGS at position 2 and to a lesser extent position 18 is conserved in AIV subtypes, but some non-H5/H7 viruses lack pGS at both sites

Sequence analysis indicated that pGS at positions 2 and 18 are conserved in all influenza viruses with prevalence rate of 98.7% (25,787/26,114) and 66.9% (17,460/26,114), respectively. The prevalence of mutations, which lead to the loss of glycosylation at the first pGS, ranged from 0.1 to 16.1% and were highest in subtype H14 (16.1%) followed by H9 (3.2%), while the first pGS was conserved in all representatives of subtypes H1, H2, H8, H11, H12 and H15 (Supplementary Table S1). While H4, H7, H9, H10 and H12-H15 had single pGS (e.g. **NYT**G), other subtypes have overlapping pGS motifs (e.g. **NNS**T). At the second position, no pGS motif was observed in subtypes H8, H9, H12, H13 and H16. The highest prevalence for the loss of the second pGS was observed in H10 viruses (0.8%), while it was completely conserved in H14 and H15. Although the pGS at position 2 was not represented in tertiary structure, however, it is expected to localize downstream from the HACS. pGS at position 18 is located upstream from the HACS and glycosylation there may sterically hinder the access of proteases (Figure 1 panel A). This sequence analysis indicates that the HA pGS at position 2 is highly conserved in most of AIV subtypes, whereas the second pGS (i.e. equivalent to position 18) was absent in five different subtypes. Some non-H5/H7, particularly H9N2, lack both pGS.

Generation of four recombinant viruses

Four recombinant viruses were successfully constructed and propagated. Virus titer was determined by plaque assay to 5×10^5 to 2×10^6 pfu/ml.

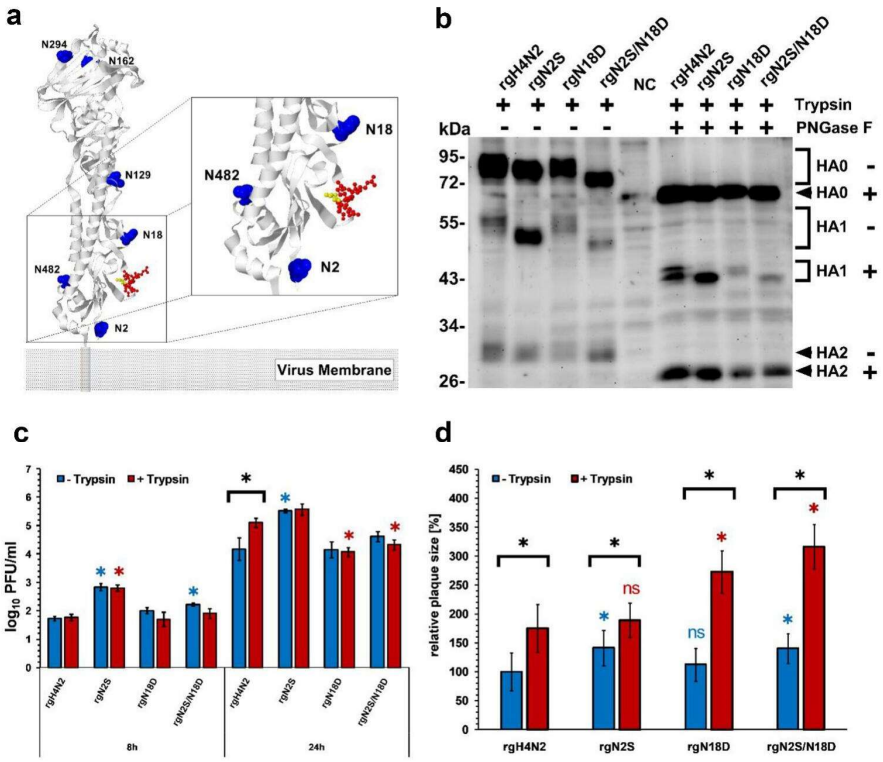

Figure 1. Structural modeling, expression, replication kinetics and cell-to-cell spread. Potential glycosylation sites are shown in blue, the CS is shown in red and threonine (T**R**/G) in the CS is in yellow. The model was generated by SWISS MODEL using the HA protein of H4N2 and further edited by Geneious. ^{2}NYT^{4} was not found in the predicted PDB-3D structure, which starts from position 5 (a). Western Blot of HA after infection of MDCK cells with (+) or without (-) treatment with PNGase F in the presence of exogenous trypsin "T". NC refers to negative control; naïve cells without infection (b). Replication kinetics in CEK cells at indicated time points after infection in the presence (T+) or absence (T-) of trypsin. Titration was done in MDCKII cells and the results are shown as mean ± standard deviation Log10 PFU/ml. Asterisks indicate significant differences at $P < 0.05$ of rgH4N2 compared to deglycosylated variants with or without exogenous trypsin (c). Cell-to-cell spread (d) was assessed by measuring 50 plaques in MDCKII with or without the addition of exogenous trypsin. Results expressed as mean and standard deviation relative to plaque size of rgH4N2 in the absence of trypsin. Asterisks indicate significant differences at $P < 0.05$ of rgH4N2 compared to deglycosylated variants with (red asterisk) or without trypsin (blue asterisk) and among each virus with or without the exogenous protease (black asterisk). ns = not significant compared to rgH4N2 with or without trypsin (D). Asterisks indicate significant differences at $P < 0.05$ of rgH4N2 compared to deglycosylated variants.

^{2}NYT^{4} and ^{18}NGT^{20} are glycosylated

Status of glycosylation was determined by treatment of the infected MDCK-cell lysates with PNGase F, which removes all N-linked glycosylation including high mannose, hybrid, bi-, tri- and tetra-antennary variants. Without PNGase F treatment, the molecular size of HA0 and HA1 of the four viruses differed, while HA2 had a similar size among all viruses indicating that variation in the size of HA0 is due to variation in the HA1 molecular weight. The HA0 of rgN2S showed a lower molecular weight compared to rgH4N2. The size of HA0 and HA1 of rgN18D were slightly lower than rgH4N2, whereas the size of rgN2S/N18D was clearly lower than rgH4N2 and rgN2S (Figure 1 Panel B). After treatment, there was no difference in the molecular weights of HA from all viruses, indicating the removal of all GS in the HA0 and a remarkably decreased shift in HA1. The HA1 of rgN2S and rgN18D had similar molecular weight indicating that the shift seen in the HA1 of rgN18D without PNGase treatment was due to glycosylation. These results indicate that both sites are glycosylated (Figure 1 panel B).

Mutation of GS at position 2 increased trypsin-independent replication of rgH4N2 in CEK

Replication of the recombinant viruses in the presence or absence of trypsin was studied in CEK. All viruses

replicated in the presence or absence of exogenous trypsin (Figure 1 panel C). The addition of trypsin increased the replication of rgH4N2 to a significantly higher level than without trypsin at 24 hpi ($p < 0.001$) (Figure 1 panel C). The addition of trypsin did not significantly affect the replication titers of the other viruses (Figure 1 panel C). Compared to rgH4N2, rgN2S replicated at significantly higher levels at 8 with or without trypsin and 24 hpi without trypsin ($p < 0.0001$) and insertion of N2S/N18D increased virus replication at significantly higher levels at 8 hpi without trypsin ($p < 0.03$) (Figure 1 panel C). N18D did not affect the replication of rgH4N2 in CEK cells (Figure 1 panel C). These results indicate that N2S increased trypsin-independent replication of rgH4N2 in CEK.

Mutation of GS at position 2 increased cell-to-cell spread with or without trypsin

To study the impact of deglycosylation on cell-to-cell spread of H4N2, plaque sizes in MDCKII cells with or without trypsin was measured (Figure 1 panel D). In the absence of trypsin, rgN2S and rgN2S/N18D produced significantly larger plaques than rgH4N2 ($p < 0.0008$). Trypsin significantly increased the size of plaques induced by each virus ($p < 0.0001$) and the largest plaques were induced by rgN18D and rgN2S/N18D ($p < 0.0001$). These data indicate that N2S increased cell-to-cell spread but trypsin is still required for efficient cell-to-cell spread of all viruses.

In MDCK-HAT and MDCK-TMPRSS2 cells all viruses replicated at similar levels

To study the possible impact of the trypsin-like enzymes HAT and TMPRSS2 on virus replication, MDCK-HAT and MDCK-TMPRSS2 cells were infected with different viruses and virus titers in collected cells and supernatant were determined by plaque assay compared to MDCK cells (which do not produce endogenous HAT or TMPRSS2) (Figure 2 panel A). In MDCK cells, all viruses replicated at similar levels, while rgN18D replicated to significantly higher titers than rgH4N2. In MDCK-HAT and MDCK-TMPRSS2 cells, all viruses replicated to comparable levels which were slightly higher than replication titers in MDCK cells, although it was not statistically significant ($p > 0.057$) (Figure 2 panel A). These data suggest that deglycosylation did not significantly affect virus replication in the presence of TMPRSS2 and HAT.

Deglycosylation reduced thermostability of rgH4N2

The impact of deglycosylation on thermostability at 50° C was studied. HA of rgH4N2 was stable for 6 hours as determined by HA assay and plaque assay (Figure 2). Conversely, deglycosylation dramatically decreased the HA activity of rgN18D and rgN2S/N18D to 82 and 63% after 30 minutes, and after 1 h both viruses had only 36 and 44% activity, respectively. From 2 hours, deglycosylation significantly decreased HA activity of rgN2S compared to rgH4N2 ($p < 0.0001$) (Figure 2 panel B). Furthermore, all viruses showed a remarkably reduced infectivity in MDCKII cells already after 30 minutes. However, the infectivity of rgH4N2 was significantly more stable after 30 minutes ($p < 0.0001$) and 1 h ($p < 0.0001$) than observed for the deglycosylated variants (Figure 2 panel C). These results indicate that glycosylation is important for thermostability of HA H4N2 at elevated temperature.

Deglycosylation reduced binding affinity to avian-type receptors

The affinity of different viruses to avian-type receptors was studied using a solid-phase binding assay against fetuin labeled with α2,3-SA. H4N2 showed strong binding affinity to avian-type receptors compared to other viruses. Human H3N2 did not bind effectively to avian-type receptors. Deglycosylation at positions 2 and/or 18 clearly reduced the binding affinity to avian-type receptors; although at higher or comparable levels to HPAIV H5N1 (Figure 2 panel D). These data indicate that deglycosylation reduced H4N2 affinity to avian-type receptors; however, the binding affinity was still comparable to HPAIV H5N1.

Deglycosylation increased cell fusion at low-acidic pH

The impact of deglycosylation on pH activation for HA-cell membrane fusion was analyzed by analysis of syncytia formation in quail fibrosarcoma (QM9, CCLV-RIE 466) cell line at different pH values between 4.0 and 6.0. While high-acidic pH (pH = 4) activation revealed no statistical difference in cell fusion among all glycosylation variants, statistical differences in syncytia formation were observed at low-acidic pH values (Figure 3). HA of rgH4N2 showed fusion activity at pH 4.0 to 5.0, while HA of rgN2S was still activated at pH values slightly higher pH 5.4. Syncytia formation for N18D and N2S/N18D was observed from pH 4.0 to 5.8. Together, deglycosylation, particularly N18D,

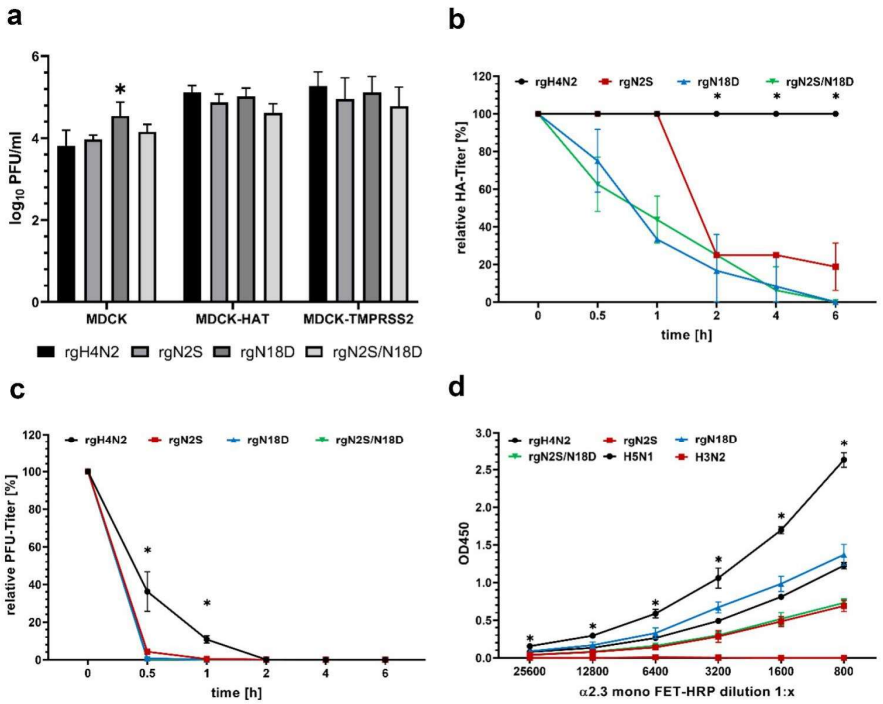

Figure 2. Impact of deglycosylation on replication, thermostability and receptor binding affinity. Multicycle replication in the cells and supernatant 24 hour post infection (hpi) of MDCK, MDCK-HAT and MDCK-TMPRSS2 cells. Asterisks indicate significant differences at $P < 0.05$ of rgH4N2 compared to deglycosylated variants (a). Heat stability at 50°C after indicated time points of all rgH4N2 variants considered in this study were tested based on HA (b) or PFU (c) titers. Asterisks indicate significant difference at P value < 0.05 of rgH4N2 compared to deglycosylated variants. Receptor binding assay after adjustment of the viruses to HA titers (d) was performed in 4 replicates to identify the affinity to the avian α2,3-linked sialic acid receptors. Avian H5N1/R65 and human H3N2 virus were used as positive and negative control, respectively.

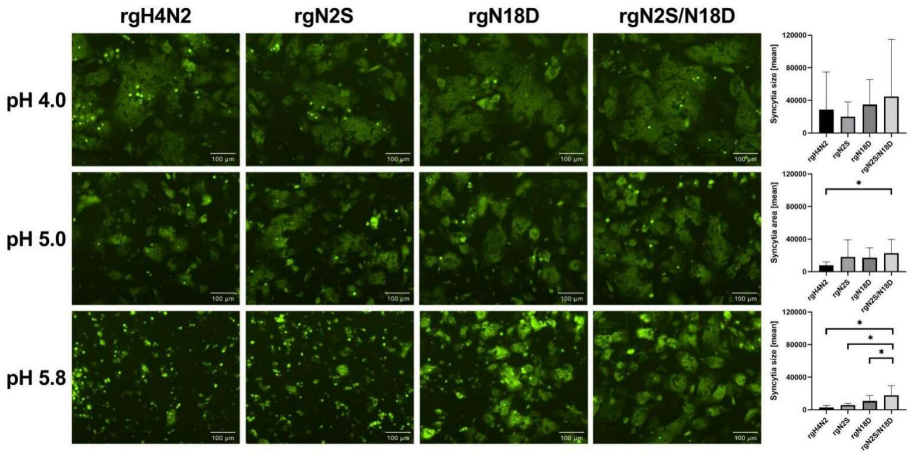

Figure 3. Impact of deglycosylation to trigger fusion at different pH values. Fluorescence microscopy of syncytia formation in QM-9 cells after expression of HA of rgH4N2 and HA carrying N2S, N18D or N2S/N18D as well as GFP, and activation at pH of 4.0, 5.0 and 5.8. Bars = 100 µm. Syncytia were measured and the average area and standard deviation for each virus is shown. Asterisks indicate significance difference at $p < 0.05$.

enabled fusion-activation of the HA at low-acidic pH conditions.

Deglycosylation expanded the tissue tropism of H4N2 in chicken embryos

To determine the histopathological changes and distribution of viruses in different tissues, organs of inoculated chicken embryos were subjected to histopathological and immunohistological examination. Necrosis in the gizzard was observed in all groups (Figure 4). Inoculation with rgH4N2 led to viral antigen detection restricted to superficial epithelia in several organs, i.e. the skin, respiratory tract (beak, trachea, lung), gastrointestinal tract (proventriculus, gizzard, small and large intestine), and bursa. Most abundantly, antigen was found in the gizzard and proventriculus (coalescent to diffuse), all other tissues exhibited focal to multifocal viral antigen. The same distribution pattern and abundancy was found for rgNS2, but additionally, focal to multifocal labeling was detected in the parenchyma of the pancreas, heart, skeletal muscle, and brain. In addition to the findings for rgNS2, rgN2S/N18D was found in the tubular epithelium of the kidney and in scattered endothelial cell in several organs. In contrast, rgN18D reflected the pattern of rgH4N2 but labeling never exceeded single positive foci. Viral antigen detection was associated with mild to moderate, acute necrosis as well as deceased cellularity within the lymphoid follicles of the bursa after inoculation with rgH4N2, rgN2S, or rgN2S/N18D. rgN18D led to mild necrosis restricted to the proventriculus and gizzard. Taken together, rgN2S particularly in combination with rgN18D led to a more widespread tissue tropism, while rgN18D alone restricted virus replication and lesions.

Deglycosylation did not alter virulence of the virus and virus excretion in chickens

Chickens were inoculated with each virus via the oculonasal (ON) route. All inoculated birds remained healthy without clinical signs or mortality. Virus excretion in OP and CL swabs at 2 and 4 dpi was analyzed by RT-qPCR (Figure 5 panels A and B). In OP swabs, the RNA was detected in all inoculated chickens except two rgN2S/N18D inoculated-chickens at 2 dpi, which were tested negative. Nevertheless, there was no significant difference in the amount of virus excretion in OP swabs between different groups (Figure 5 panel A). Cloacal shedding was determined in low amounts, if at all, on both days (Figure 5 panel B). In CL swabs, at 2 dpi, the viral RNA was detected in inoculated chickens with rgH4N2 (2/5), rgN18D (1/5) and rgN2S/N18D (3/5). Conversely, at 4 dpi, rgN2S was detected in 3/5 birds and only 1/5 birds in other groups were positive. Trials to re-isolate the virus or direct sequencing in swab samples in this experiment were not successful. All inoculated chickens possessed anti-NP antibodies as

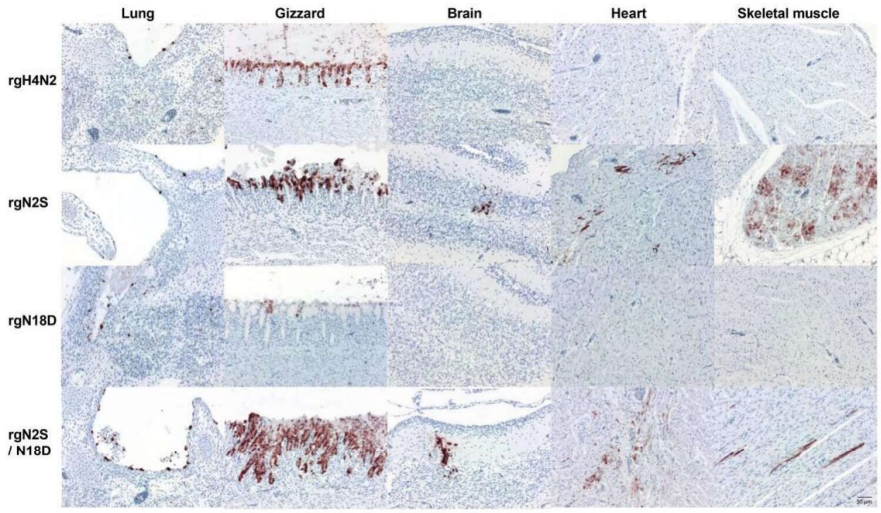

Figure 4. Distribution of avian influenza virus matrix protein (M1) in selected organs of chicken embryos. Distribution of influenza M1 protein in the indicated organs of embryos inoculated with different viruses. 14-day-old embryonated chicken eggs were inoculated via the allantoic sac for 5 days then embryos were subjected to immunohistochemistry using primary anti-matrix antibody for antigen detection. Labeling by 3-amino-9-ethyl-carbazol (red-brown); hematoxylin counterstain (blue). Bars = 50 µm.

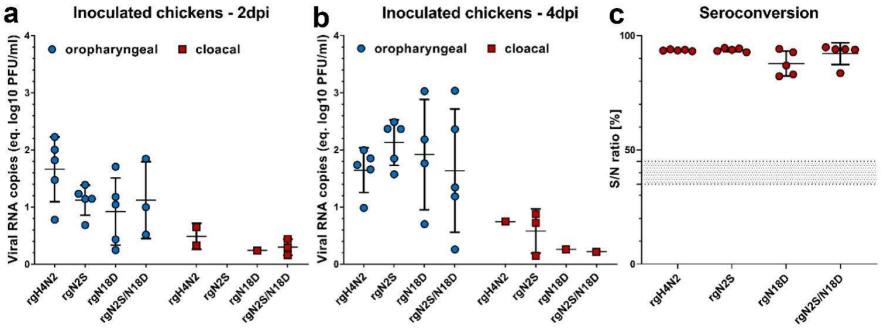

Figure 5. Virus excretion in oropharyngeal and cloacal swabs and seroconversion after intranasal inoculation of chickens. Virus excretion in oropharyngeal and cloacal swabs collected at 2 (a) and 4 (b) dpi in inoculated chickens was determined by RT-qPCR targeting the M gene. Results are shown as viral RNA copies (equivalent log10 PFU/ml) with average ± standard deviation. Anti-NP antibodies were determined by inhibition ELISA in serum samples collected from all chickens at the end of the experiment (c). Shown are the average S/N ratio and standard deviation of positive samples.

tested by ELISA (Figure 5 panel C). These results indicate that deglycosylation around the HACS did not significantly affect low virulence or excretion of the H4N2 with a unique natural polybasic cleavage site in chickens.

Discussion

HPAIV H5 and H7 evolve from LP precursors after mutation of the monobasic HACS to a polybasic motif. Non-H5/H7 viruses with a polybasic CS are very rare in nature. In 2012, a unique H4N2, the only non-H5/H7 with 4 basic aa (^{322}PEKRRTR/G^{329}) in the HACS was isolated from quails, but the virus exhibited low virulence in chickens [22]. In contrast to H5 and H7 viruses, no data are available on the role of glycosylation in the HA1 N-terminus of non-H5/H7 viruses. Whether removal of conserved GS in the vicinity of the HACS affects virus fitness and subsequently replication and virulence in chickens was studied herein.

In this study, sequence analysis of 26,114 HA proteins obtained from GenBank and GISAID showed that pGS at positions 2 and 18 were conserved across AIV HA subtypes, particularly those adapted to poultry, except H9 viruses which lack glycosylation at position 18 which is partially similar to a previous report [37]. Remarkably, more than 240 H9 viruses lack pGS at both sites indicating that they might be dispensable for replication. We showed that asparagine at positions 2 and 18 are glycosylated which is in accordance with results on the analogous positions in some H5 and H7 viruses [12,15,18]. It is known that trypsin and trypsin-like enzymes are important for multiple cycle replication of LPAIV. However, rgH4N2 in this study replicated to a certain level in CEK cells and spread from cell-to-cell in MDCKII without trypsin. Both cell lines have endogenous proteases (e.g. matriptase) which may activate some LPAIVs [38]. Moreover, furin-like enzymes or proteases in the allantoic fluid during propagation of viruses in ECE probably supported the growth of rgH4N2 without trypsin as previously described [39]. It is worth to mention that trypsin-independent replication of LPAIV H6N1 and H7N7 in MDCK, MDCKII and/or CEK cells has been previously reported [20,40]. Deglycosylation of N2S (alone or in combination with N18D) increased virus replication in CEK cells and cell-to-cell spread in MDCKII cells without trypsin. Conversely, the removal of a similar GS ^{11}NST^{13} in an HPAIV H5N1 or HPAIV H7N1 impaired activation of HA0 and decreased virus growth in cell culture [18,41]. Glycosylation at position 28 (equivalent to N18D in H4N2) was indispensable for the infectivity of HPAIV H7N1 [41].

HA0 is a fusion-inactive protein which must be cleaved by host proteases into the HA1/HA2 two subunit complex to expose the fusion peptide in the HA2 [7]. The fusion of influenza viruses with host cell membranes is pH-dependent which results in irreversible conformational changes. The fusion alters intracellular host cell responses (e.g. IFN response) and subsequently regulates infectivity. The pH value of the early endosome is about 6 to 6.3 and in the late endosome is 5 to 6 (reviewed by [42]). Thus, rapid fusion may enhance early virus replication before triggering the host-immune response. However, pH stability is also important for persistence in the environment. Therefore, AIV vary in their optimal range for pH-triggered fusion/activation from 4.4 to 6.4 [42,43]. In this study, rgH4N2 showed optimal pH-triggered activation at high acidic pH (pH ≤ 5). In the presence of trypsin, the N18D mutation alone or in combination with N2S triggered syncytia formation at low acidic pH

(5.8). Similar observation for pH fusion activation has been reported after removal of GS in position 28 (equivalent to position 18 in H4N2) in the HA stem domain of A/FPV/Rostock/34 (H7N1) [15]. Hence, the GS at position 18 is located closer to the middle of the HA stem domain than the site at position 2. It seems possible that oligosaccharides at position N18 are required for stabilization of the metastable HA during virus-host membrane fusion [15]. Moreover, it is known that glycosylation is important for stability of the HA protein especially at higher temperature (e.g. intestinal tract of birds, fever or at ambient temperature) [44]. In this study, mutations N2S or N18D dramatically affected HA activity and infectivity. Similar results were observed for A/Mallard/Huadong/S/2005 H5N1 after removal of the glycosylation at position ^{11}NST^{13} indicating an impact on structural stability by glycosylation at the N-terminus [18]. Interestingly, H4N2 had a stronger affinity to α2,3 SA receptors than H5N1. Deglycosylation at positions 2 and 18 significantly reduced binding affinity to α2,3 SA indicating conformational changes in the head domain. Similar results have been described after deglycosylation at ^{11}NST^{13} of an H5 virus [45]. However, this reduction in receptor affinity was similar to HPAIV H5N1 and apparently sufficient for efficient virus replication in chicken cells or embryos.

Chicken embryos as a model have been previously used to assess tissue distribution and virulence of different influenza viruses [35,46–48]. In this study, the rgH4N2 viral antigen distribution was restricted to superficial epithelia mainly in the respiratory and gastrointestinal tract and was associated with necrosis. Deglycosylation N18D restricted the viral tropism and lesions whereas deglycosylation N2S or N2S/N18D led to a more systemic spread and lesions profile, also affecting the brain, heart, skeletal muscles and kidneys. These data indicate that deglycosylation N2S is advantageous for systemic virus tropism in chicken embryos. In chickens, rgH4N2 exhibited low virulence despite of the polybasic cleavage site which is similar to previous findings [22,25]. In contrast to these results, deglycosylation at position 11 (equivalent to position 2 in H4N2) reduced virulence of HPAIV H5N1 in intravenously inoculated 6-week-old chickens [18].

In conclusion, H4N2 with a unique polybasic cleavage site and mutation of GS at position 2 and 18 exhibited increased trypsin-independent replication in chicken cells, increased cell-to-cell spread, triggered fusion activation at low acidic pH and enabled systemic spread in chicken embryos, which were driven mostly by the N2S mutation. Both mutations reduced receptor binding and thermostability. No impact on virulence in chickens was observed, however. Despite the important role of glycosylation in the vicinity of H4N2 HACS for HA stability, removal of the glycosylation at position 2 may play a role in adaptation of non-H5/H7 which is in contrast to similar studies using HPAIV H5N1 or H7N1.

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Disclosure statement

There are no relevant financial or non-financial competing interests to report

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Supplementary Table S1: Prevalence of potential N-glycosylation in the N-terminus of HA1 in different AIV subtypes

Subtype Hx	**N2***	**N18**
H1	**100%** (808/808)	**99.6%** (811/814)
H2	**100%** (504/504)	**99.8%**(503/504)
H3	**98.9%** (1832/1852)	**99.5%** (1882/1892)
H4	**98.1%** (1424/1452)	**99.9%** (1461/1463)
H5	**99.9%** (6881/6886)	**99.7%** (6925/6948)
H6	**99.9%** (2012/2014)	**99.9%** (2019/2021)
H7	**99.2%** (2220/2239)	**99.5%** (2228/2240)
H8	**100%** (204/204)	**0%** (0/204)
H9	**96.8%** (7415/7657)	**0%** (0/7657)
H10	**99.5%** (772/776)	**99.2%** (777/783)
H11	**100%** (811/811)	**99.8%** (809/811)
H12	**100%** (335/335)	**0%** (0/335)
H13	**99.7%** (363/364)	**0%** (0/364)
H14	**83.9%** (26/31)	**100%** (31/31)
H15	**100%** (14/14)	**100%** (14/14)
H16	**99.4**% (166/167)	**0%** (0/167)
Total	**98.7% (**25787/26114)	**66.9%** (17460/26114)

* Percent of number of sequences with a potential N-glycosylation site/total number of sequences retrieved from GISAID and GenBank to 27-01-2020. The prevalence of N-linked glycosylation at the first two positions in HxNx viruses (equivalent to H4 positions N2 and N18) was predicted by N-X-S/T motif, where X is any amino acid except proline.

II

(II) Insertion of Basic Amino Acids in the Hemagglutinin Cleavage Site of H4N2 Avian Influenza Virus (AIV)-Reduced Virus Fitness in Chickens is Restored by Reassortment with Highly Pathogenic H5N1 AIV

Marcel Gischke, Reiner Ulrich, Olanrewaju I Fatola, David Scheibner, Ahmed H Salaheldin, Beate Crossley, Eva Böttcher-Friebertshäuser, Jutta Veits, Thomas C Mettenleiter and Elsayed M Abdelwhab

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Insertion of Basic Amino Acids in the Hemagglutinin Cleavage Site of H4N2 Avian Influenza Virus (AIV)—Reduced Virus Fitness in Chickens 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 hemagglutinin (HA) and any AIV with an intravenous pathogenicity index (IVPI) ≥ 1.2. Although only a few non-H5/H7 viruses fulfill the criteria of HPAIV; it remains unclear 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 HPAIV 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 treatment with trypsin, particularly in MDCK cells, and reassortment with HPAIV H5N1. 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 seven 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 HPAIV 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

1. 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 10 proteins in addition to strain specific non-structural accessory proteins [1,3]. 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 [4]. AIVs are further classified according to virulence in chickens into low pathogenic (LP) and highly pathogenic (HP) pathotypes. LPAIV induce only mild or no clinical signs, while HPAIVs cause severe illness with mortality rates up to 100% within a few days [5]. HPAIVs evolve from LP progenitors after circulation in domesticated birds and are naturally restricted to H5 and H7 subtypes. The transition 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 [6]. The HA protein is synthesized as a fusion-inactive precursor (HA0) that requires processing by the 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 (AAs) arginine (R) and/or lysine (K) is a major virulence factor [7,8]. 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 a monobasic cleavage site. However, it remains to be investigated whether orthologous proteases (e.g., HAT and TMPRSS2) of birds also support HA cleavage in birds. Conversely, the polybasic CS of HPAIV is cleaved by ubiquitous, subtilisin-like proteases causing systemic infection and multiorgan dysfunction [9].

Despite carrying polybasic CS motifs, some H5 and H7 viruses exhibited low virulence in chickens [6,10]. The virulence of several of these viruses was enhanced by increasing the numbers of basic AAs in the CS or by additional mutations in the HA or other gene segments [11–13]. Interestingly, a few natural non-H5/H7 viruses also fulfill the criteria of HPAIV. Several H10Nx viruses with monobasic CS exhibit an intravenous pathogenicity index (IVPI) higher than 1.2 resembling the HPAIV H5/H7 [14–16]. Moreover, in August 2012, an H4N2 virus was isolated from quails in California that possessed the polybasic CS motif ^{322}PEKRRTR/G^{329} [17]. It is the only non-H5/H7 virus with four basic AAs in the CS, which complies with the HPAIV furin-specific cleavage motif R-X-X-R. Although this virus replicated and transmitted efficiently in chickens, it did not cause morbidity or mortality [17]. It has been reported that some H5/H7 viruses possessed "intermediate" polybasic CS, which evolved stepwise to accumulate an increasing number of basic AAs, due to strand slippage induced by RdRp [18] or a predisposing RNA secondary structure [19], as found in HPAIV H5N2 in Mexico in 1994 [20], H7N7 in Chile in 2002 [21], H7N7 in Canada in 2004 [22] and even the circulating H5N1 Goose Guangdong (Gs/Gd) virus since 1996/1997 [23]. 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 [13,24,25]. 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 [26]. It has been shown that non-H5/H7 viruses are capable of shifting to high virulence after acquisition of a polybasic CS [27,28] and other gene segments from HPAIV H5N1 [29]. However, little is known about the resulting impact on viral fitness to explain the lack of expansion of non-H5/H7 HPAIV in birds.

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

2. Results

Beside the recombinant H4N2_wt specifying CS ^{322}PE**KRRTR**/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}) combined with the other seven gene segments from H4N2, HPAIV H5N1 or HPAIV H7N7 were successfully generated using reverse genetics (Table 1). Trials to generate an H4N2 carrying a 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 glutamic acid (^{322}PQ**RRR**E**KKR**/G^{331}) in the HACS.

Table 1. Recombinant viruses generated in this study.

Virus	Cleavage Site	Source of	
		HA	Other Gene Segments
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**QRRREKK**R/G^{331}	H4N2	H4N2
H7N7_HA4	^{322}PEKRRTR/G^{329}	H4N2	H7N7
H5N1_HA4	^{322}PEKRRTR/G^{329}	H4N2	H5N1 (Gs/Gd)
H5N1_HA4_T^{327}R	^{322}PEKRR**R**R/G^{329}	H4N2	H5N1 (Gs/Gd)
H5N1_HA4_T^{327}K	^{322}PEKRR**K**R/G^{329}	H4N2	H5N1 (Gs/Gd)

Residues 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. * Trials to generate an H4N2 carrying a HPAIV H5N2 HACS (^{322}PQ**RRRKKR**/G^{330}) were not successful. After several rescue attempts, a virus was obtained with a spontaneous insertion of glutamic acid (^{322}PQ**RRR**E**KKR**/G^{331}) in the HACS. Gs/Gd = Goose Gunagdong H5N1 lineage.

2.1. The Expansion of the Polybasic CS in H4N2 Virus had a Minimal Impact on Virus Replication in Cell Culture but the Replication of the H4N2 Virus Was Significantly Increased by Reassortment with H5N1 or H7N7

Virus replication was studied after the 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 virus titers at 24 and 48 hpi, although the difference 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 irrespective of the presence 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). Replication of H4N2_H5N2-HACS was significantly reduced compared to H4N2_wt (Figure 1). H7N7_HA4 carrying the HA from H4N2_wt and the other seven gene segments from HPAIV H7N7 replicated to significantly higher levels than H4N2_wt at 8, 24 and 48 hpi (Figure 1). Moreover, the three viruses carrying seven gene segments from HPAIV H5N1 replicated to significantly higher titers than H4N2_wt particularly at 24 hpi, however, expansion of the HACS did not alter the replication level in this panel of reassortments in CEK (Figure 1). In MDCK cells, H4N2 viruses carrying authentic HA, HA_T^{327}R or HA_T^{327}K showed no replication at 8 hpi without trypsin and only H4N2_HA_T^{327}K produced viral progeny at very low titers in the presence of 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 the replication of H5N1_HA4 to similar levels as H5N1_T^{327}R/K (Figure 2). Moreover, the H5N1 virus replicated in MDCK cells at higher levels than H4N2 viruses and trypsin had no significant impact on H5N1 virus replication ($p > 0.49$). Together, the replication of H4N2 viruses was

enhanced by the addition of trypsin particularly in MDCK cells and reassortment with HPAIV H5N1 or H7N7 segments in CEK.

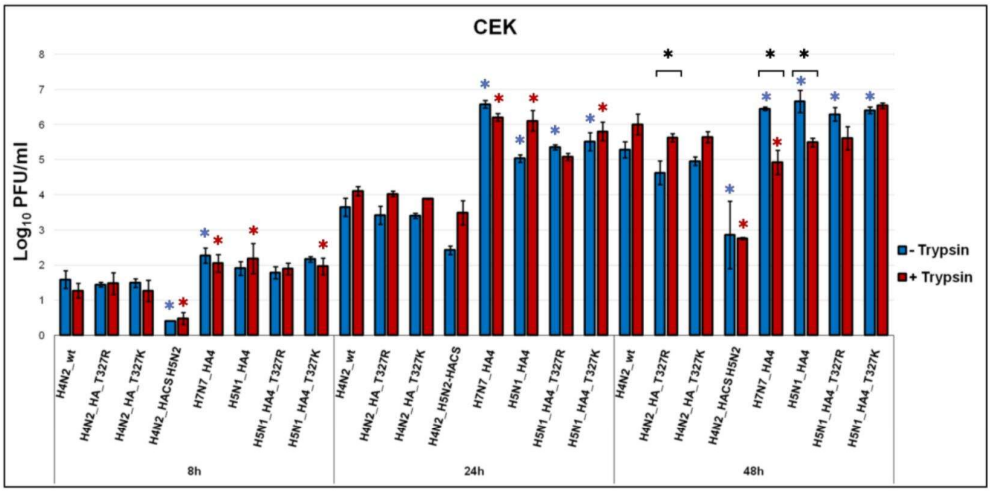

Figure 1. Replication kinetics of recombinant viruses in chicken embryo kidney cells (CEK). Replication kinetics in CEK cells at indicated time points after infection with H4N2 viruses with variable HACS or with H7N7 and H5N1 gene segments with (T+) or without (T−) trypsin. Titration was done in MDCKII cells and the results are shown as mean ± standard deviation Log_{10} PFU/mL. Asterisks indicate significant difference ($p < 0.05$). Blue and red asterisks indicate significant differences compared to H4N2_wt without or with trypsin, respectively. Black asterisks indicate significant differences for replication of each virus in the presence or absence of trypsin.

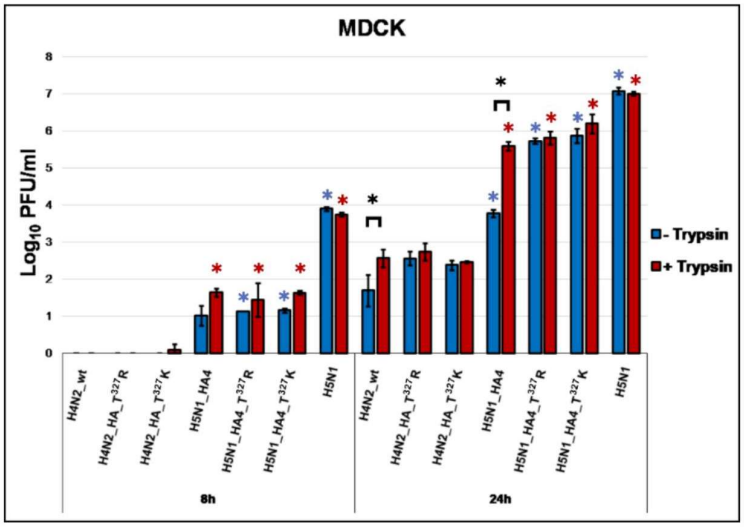

Figure 2. Replication kinetics of recombinant viruses in Madin–Darby canine kidney cells (MDCK). Replication kinetics in MDCK cells at indicated time points after infection with H4N2 viruses with variable HACS or H5N1 gene segments with (T+) or without (T−) trypsin. Titration was done in MDCKII cells and the results are shown as mean ± standard deviation Log_{10} PFU/mL. Asterisks indicate significant difference ($p < 0.05$). Blue and red asterisks indicate significant differences compared to H4N2_wt without or with trypsin, respectively. Black asterisks indicate significant differences for replication of each virus in the presence or absence of trypsin.

2.2. 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 three days. In MDCKII cells, all viruses were tested without the addition of trypsin except for H4N2_wt and H4N2_H5N2-HACS. The H4N2_wt virus produced plaques in MDCKII irrespective of the presence of trypsin, although the addition of trypsin significantly increased the size of plaques (Figure 3A). The spread of H4N2_T^{327}R/K or H5N1_HA4 from cell-to-cell was comparable to H4N2_wt in the absence of trypsin. However, H4N2_H5N2-HACS produced significantly smaller plaques without trypsin while in the presence of trypsin they were larger than those induced by H4N2_wt (Figure 3A). The plaque size of H4N2_T^{327}R or H4N2_T ^{327}K significantly increased by 52% and 125% in combination with the other seven H5N1 segments (Figure 3A). In MDCK cells, the addition of trypsin increased the plaque size of all viruses. Viruses carrying H5N1 segments produced larger plaques than H4N2_wt, particularly when grown in medium containing trypsin (Figure 3B). Furthermore, the H5N1 virus produced significantly larger plaques in MDCKII and MDCK cells compared to H4N2_wt particularly in the absence of trypsin. Notably, trypsin increased cell-to-cell spread of all viruses in MDCK cells. Taken together, the expansion of the polybasic CS had a minimal impact on virus spread in cell culture but the replication of H4N2 virus was significantly increased by reassortment with H5N1 and/or the addition of trypsin.

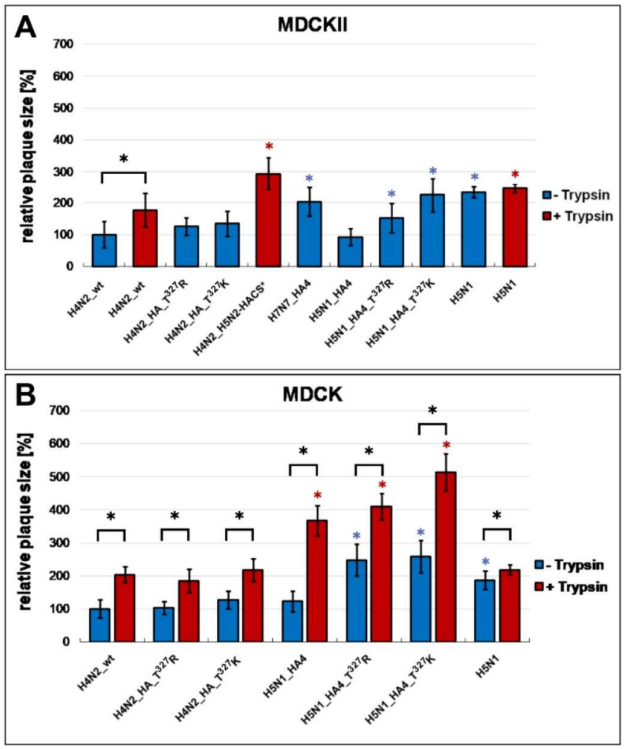

Figure 3. Cell-to-cell spread in MDCKII and MDCK cells. Cell-to-cell spread was assessed by measuring 50 to 100 plaques in MDCKII (**A**) or MDCK (**B**) cells with (T+) or without (T–) trypsin. In MDCKII (**A**), trypsin was added only to H4N2_wt and H4N2_H5N2-HACS. The latter virus did not produce plaques without trypsin. Results are expressed as mean and standard deviation relative to the plaque size of H4N2_wt in the absence of trypsin. Asterisks indicate significant differences ($p < 0.05$); blue and red asterisks indicate significant differences compared to H4N2_wt without or with trypsin, respectively. Black asterisks indicate significant differences for the replication of each virus in the presence or absence of trypsin.

2.3. T^{327}R/K Enabled Partial Trypsin-Independent Activation of the HA by Endogenous Furin-Like Protease

To determine the impact of expansion of the CS on HA cleavage, 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 the addition of trypsin increased cleavability (Figure 4A). Furthermore, 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 still cleaved. The cleavage of HA was inhibited using MI-1148. HAT and TMPRSS2 failed to activate HA_T^{327}K (Figure 4B). The expression of TMPRSS2 and HAT in HEK293T cells was confirmed using a Western Blot. Altogether, T^{327}R/K enabled partial activation of the HA in the absence of trypsin by a yet to be identified endogenous furin-like protease.

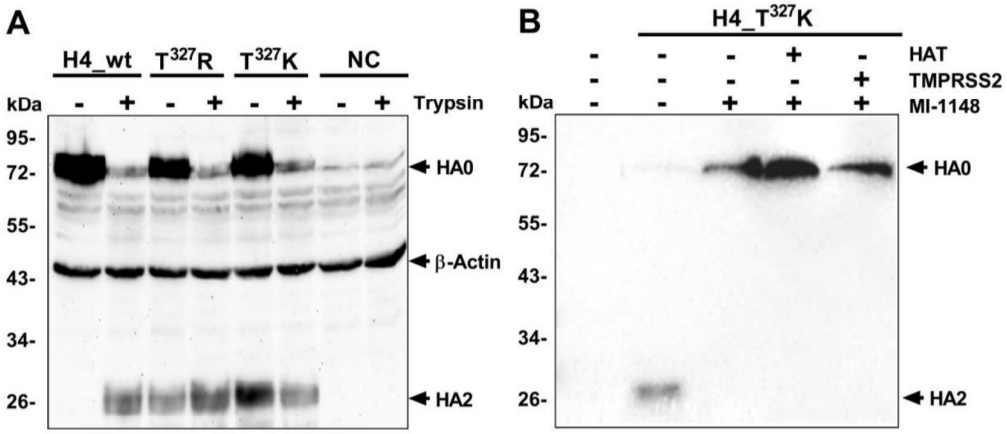

Figure 4. Cleavability of the HA after transfection of HEK293T cells. Cleavability of the HA of H4N2_wt or HA with R^{327} or K^{327} in the presence or absence of exogenous trypsin tested with a Western Blot using β-Actin as internal controls (**A**). The cleavability of HA of H4N2_T^{327}K in HEK293T 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 a lack of activation of HA0 by TMPRSS2 and HAT (**B**). NC refers to the negative control; naïve cells without transfection or infection.

2.4. The Expansion of the Cleavage Site Alone Was Not Enough for Exhibition of High Virulence after ON (Oculonasal) 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 a PI of 0 (Table 2). All experimentally infected birds in these groups seroconverted at the end of the experiment, except for one chicken inoculated with H4N2_H5N2-HACS (Table 2). While all sentinels in-contact with 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 exhibited seroconversion (Table 2), 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 two 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 the clinical examination after the 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 an 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 the H4N2_T^{327}R injected chickens showed morbidity or mortality with IVPI values of 0. The H4N2_T^{327}K group exhibited moderate virulence with an IVPI of 0.6 and moderate to severe neurological disorders (e.g., torticollis, opisthotonos and 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 several 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 the exhibition of the high virulence of the H4N2 virus. T^{327}K and H5N2_HACS compromised virus transmission as indicated by the lower number of contact birds with AIV antibodies.

2.5. Virus Excretion in Inoculated and In-Contact Chickens Was Reduced by T^{327}R/K and Increased by Reassortment with HPAIV H5N1 Segments

The H4N2_wt was detected at 4 dpi 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 5A). Both viruses were not detected in swabs in contact birds further indicating the negative impact on virus transmission (Figure 5B). 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 5A,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 5A,B). H5N1_HA4_T^{327}R was detected in oropharyngeal (n = 1/2) and cloacal (n = 2/2) swabs (Figure 5A). As H5N1_HA4_T^{327}K killed all inoculated birds within two days, it was not possible to collect swabs at 4 dpi in this group. Both H5N1_HA4_T^{327}R and H5N1_HA4_T^{327}K were excreted from all contact birds (Figure 5B). 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. Reassortment with segments from HPAIV H5N1 increased virus excretion in oropharyngeal and cloacal swabs.

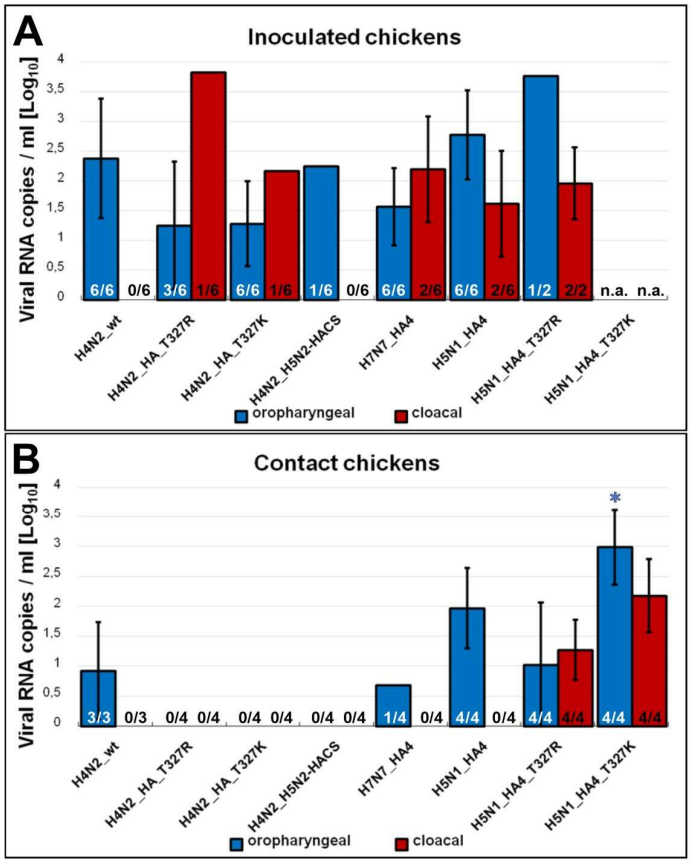

Figure 5. Virus excretion from oropharyngeal and cloacal swabs of inoculated and sentinel chickens. Virus excretion in oropharyngeal and cloacal swabs in inoculated (**A**) and contact (**B**) birds was determined by RT-qPCR targeting the M gene. The averages ± standard deviation of viral RNA copies/ml and number of positive birds/total examined are shown. Samples were collected at 4 dpi from all surviving birds. n.a. = not applicable because all birds inoculated with H4N2_T^{327}K died at 2 dpi.

2.6. 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 virus distribution in different tissues, organs of at least two inoculated chickens per group were subjected to histopathological and immunohistological examination for the detection of the 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, the 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 oligofocal distribution 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 6A,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 the 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, necrotizing myocarditis were observed. The NP of H5N1_HA4_T^{327}K was detected in the endothelial and parenchymal cells of almost all organs (Figure 6A,B) and the intensity ranged from median scores of 0.5 in the hepatic endothelium as well as in the 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 the 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 a 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 more widespread than viruses carrying T^{327}R (Figures 6 and 7), except for lymphoid depletion.

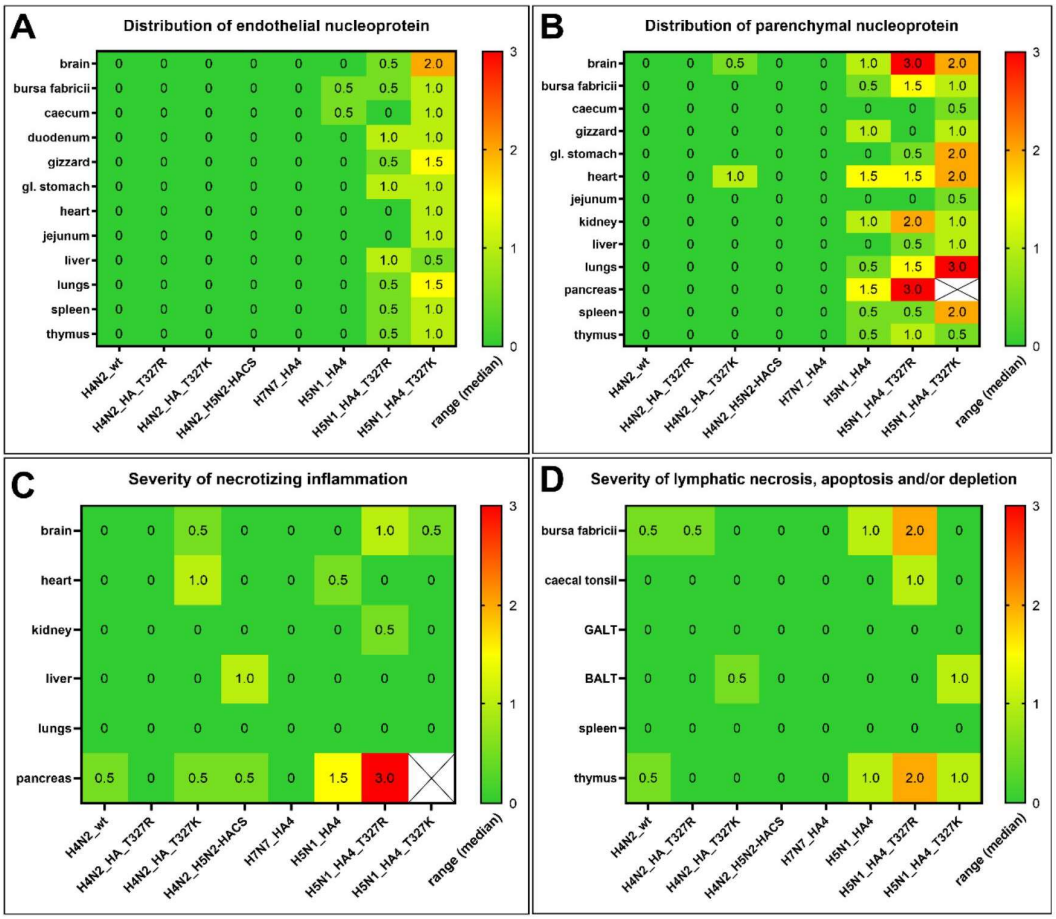

Figure 6. Distribution of avian influenza virus NP in organs of inoculated birds and severity of lesions. Distribution of NP antigen in endothelial (**A**) and parenchymal (**B**) cells as well as the severity of necrotizing inflammation (**C**) and lymphatic depletion (**D**) in the affected organs of inoculated birds scored from 0 to 3.0 (green to red color). Results are shown as the 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 were kept in the refrigerator until 4 dpi. Pancreas samples with white cells and an X symbol in panels B and C were not tested because of insufficient quality for evaluation.

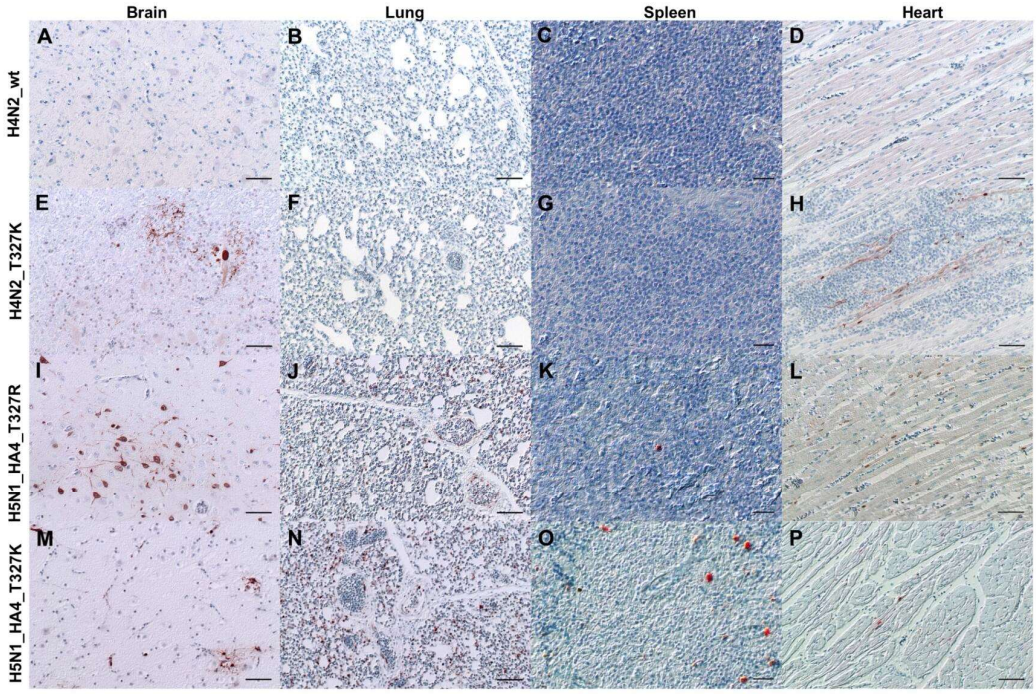

Figure 7. Distribution of avian influenza virus NP in selected organs of inoculated birds. Distribution of influenza NP in brain (**A**,**E**,**I**,**M**), lung (**B**,**F**,**J**,**N**), spleen (**C**,**G**,**K**,**O**) and heart (**D**,**H**,**L**,**P**) of inoculated chickens of selected viruses at 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.

3. Discussion

Wild birds represent the natural reservoir for LPAIV. HPAIVs evolve from LPAIV 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 [17]. 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 restricted 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 AAs or removal of a glycosylation site in the vicinity of the CS [30]. Interestingly, the current H4N2 virus has potential glycosylation sites in the vicinity of CS in the HA1 [17], resembling H5N2/1983, which hinder cleavage by different proteases [31,32]. Moreover, we showed that furin-like protease(s) can also cleave the HA of H4N2_T^{327}K in transfected HEK293T independent of the presence of HAT and TMPRSS2 that activate some viruses with monobasic CS [33] and some H9N2 viruses with monobasic VSSR/G, dibasic RSSR/G or tribasic RSRR/G cleavage site motifs [32]. Interestingly, these viruses were not activated by furin without further insertion of basic AAs at the CS despite matching the minimal consensus sequence [27,34]. Furthermore, Wong et al. [17] showed that replication 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. MDCKII cells are a natural subclone of MDCK cells and both cell lines are commonly used for characterization of AIV. It has been reported that MDCKII and CEK cells have matriptase, which is not present in the MDCK cells [32]. Matriptase activated H9N2 AIVs with R-X-X-R or R-X-R-R motifs [32], 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 [28,35]. 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 [36]. Moreover, reassortment with the H5N1 segments increased H4N2 virus spread more effectively in MDCK cells. H4N2_wt possessed E627, while HPAIV H5N1 possessed K627 in PB2 [37], which is known to increase the polymerase activity and replication of H5N1 virus in mammal cells [37].

Successful replication of an AIV in poultry is a prerequisite for progressive adaptation including efficient bird-to-bird transmission and high virulence [38]. 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 AAs in the CS on virus replication and transmission in chickens probably precludes their expansion in nature. Moreover, the increased number of basic AAs in the presence of other gene segments from H4N2 did not result in an HP phenotype after ON or IV infections [24,35]. In other studies, the insertion of a polybasic CS conferred high virulence to a low-pathogenic H6N1 virus (IVPI = 1.4) [28] but not an H3N8 virus [39]. 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 [11,12,40]. H9N2 with polybasic CS and gene segments from HPAIV H5N1 exhibited a low-level HP phenotype (IVPI = 1.23) [27]. 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 [29]. Remarkably, high virulence was not observed after the 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 [41]. Conversely, for the current HPAIV H5N1, in addition to the polybasic CS, a deletion within the NA stalk domain (which is also present in the H4N2 virus [17]) and the presence of autologous polymerase genes were important for high virulence in chickens [11]. Furthermore, compared to the H4N2 virus, HPAIV H5N1 used in this study possessed residues in the NP (S377N) [42], NS1 (deletion of AAs 80–84) [43] and PB1 (V14A) [44]. These residues were linked to higher virulence or transmission of H5N1 viruses in chickens, which remain to be investigated. Similarly, it has been also shown that residues, yet to be identified, in PB2, PB1 and NP affect the high virulence of HPAIV H5N1 in chickens [12].

It is known that the presence of a polybasic CS increases the dissemination of HPAIV H5/H7 in different organs causing multiorgan dysfunction and death [7,15,26,28,39]. We showed here that a polybasic CS alone was not sufficient 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 [45,46]. Also, the M2 protein protects the HA from premature conformational changes increasing the stability of influenza viruses [47]. Therefore, it is important to further determine the specific gene segment(s) of H5N1 that support the HP phenotype of H4N2 virus.

Another finding was that $T^{327}K$ was advantageous over $T^{327}R$; it increased 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 [48]. 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 [49]. Intriguingly, the majority of HPAIV H5/H7 possessed lysine at position P2 [23], resembling the $T^{327}K$ in this study, which may support our assumption. Remarkably, the pathology of H5N1_HA4_$T^{327}R$ was more severe in the bursa, thymus, pancreas and brain compared to H5N1_HA4_$T^{327}K$ and H5N1_HA4. This is probably due to the observed higher lymphatic depletion and/or efficient replication of the virus in these organs.

In conclusion, the insertion of additional basic AAs 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, the expansion of HPAIV H4N2 in nature is unlikely. Although it remains speculative, the evolution of natural HPAIV H4N2 will require prior reassortment with HPAIV H5N1-like gene segments to achieve a higher fitness 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.

4. Materials and Methods

4.1. 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, USA). 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 [11]). Plasmids containing eight gene segments of HPAIV A/chicken/Germany/AR1385/2015 (H7N7) were described [41]. pCAGGS plasmids encoding HAT and TMPRSS2 have been reported previously [50].

Primary chicken embryo kidney (CEK) cells used for the determination of replication kinetics were prepared according to standard procedures [51]. 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.

4.2. 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, Helden, Germany). All eight genomic segments of the H4N2 virus were amplified by specific primers and cloned into the pHW*SccdB* plasmid [52]. 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, Carlsbad, CA, 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 [53]. 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 the pCAGGS vector to increase protein expression.

4.3. 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) [54]. 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 a hemagglutination test using 1% chicken erythrocytes according to the OIE recommended protocol [54]. AF with a titer >16 (4log$_2$) hemagglutination units was checked for bacterial contamination by streaking sheep blood agar at 37 °C for up to 72 h. 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. All gene segments of all viruses were sequenced to exclude unwanted mutations by Sanger sequencing using ABI BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA USA). The H4 AA numbering is based on the mature protein after removal of the signal peptide.

4.4. 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 1× phosphate buffered saline (PBS) and covered with minimum essential medium (MEM) containing 0.2% bovine serum albumin (BSA) (MP Biomedicals, Eschwege, Germany). Cells infected with recombinant H4N2_wt were grown in the presence or absence of 2 µg/µL of *N*-tosyl-ʟ-phenyalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma Aldrich, Steinheim, Germany). Plates were incubated at 37 °C and 5% CO_2. Cells and supernatant were harvested at the indicated time points post-infection (hpi) and stored at −80 °C. Virus titers were determined by the plaque assay as described below. The replication kinetics were run in duplicates and repeated three times. Results are expressed as average values and the standard deviation was indicated for all replicates.

4.5. Plaque Assay

Confluent MDCKII or MDCK cells in 6-well plates were washed once with PBS and incubated with 10-fold serial dilutions of propagated viruses or samples for 1 hour at 37 °C in a 5% CO_2 atmosphere. Thereafter, cells were washed twice with 1× PBS and covered by semi-solid Bacto^{TM} Agar (BD, Pont-de-Claix, France) with 50% MEM containing 4% BSA (MP Biomedicals, Eschwege, Germany). All plates were incubated for 3 days at 37 °C in 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 h. 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, Amsterdam, Netherlands). The 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.

4.6. Western Blot

HA cleavability was assessed in HEK293T cells in the presence or absence of exogenous proteases (i.e., trypsin, TMPRSS2 and HAT) using standard Western Blot procedures with few modifications [55]. The cleavage of HA of H4N2_wt, H4N2_T^{327}R and H4N2_T^{327}K in the presence or absence of 2 µg/mL

TPCK-treated trypsin was studied by transfecting cells with 5 µg pCAGGS plasmid coding for HA of the different viruses using Lipofectamine 2000 transfection reagent (ThermoFischer Scientific, Karlsruhe, Germany). The transfected cells were incubated with MEM containing 0.2% BSA at 37 °C in 5% CO_2 for 24 h. For TMPRSS2 and HAT, HEK293T cells that do not express an endogenous HAT or TMPRSS2 [56], were co-transfected with 1 µg pCAGGS plasmids containing H4N2_T^{327}K, as well as 10 ng plasmid coding for each protease in the presence or absence of 50 µM furin inhibitior MI-1148 (kindly provided by Torsten Steinmetzer, Institute of Pharmaceutical Chemistry, Philipps-University Marburg, Marburg, Germany) [50]. After 48 h, transfected cells were harvested, washed with PBS and sedimented at 14,000× *g* for 15 min. 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 1 h in 5% skimmed 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, Cambridgeshire, UK) for β-Actin. The immunodetection was done by chemiluminescence using Clarity™ Western ECL Substrate (BioRad, Feldkirchen , Germany). Images were captured by a Bio-Rad Versadoc 4000 Molecular Imager (BioRad, Munich, Germany) and Quantity One software (BioRad, Munich, Germany).

4.7. Animal Experiments

All animal experiments 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, permission number 7221.3-1.1-051-12). The commissioner for animal welfare at the FLI representing the Institutional Animal Care and Use Committee (IACUC) approved all experiments (TV04/17; December 2016).

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-weeks-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 0.1 mL 1:10 diluted AF according to the OIE standard protocol [54]. All birds were observed daily for clinical signs and mortality for 10 (IV) or 14 (ON) dpi. The severity of clinical signs was assessed using a standard pathogenicity index (PI) as recommended [54]. 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 termination 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, Düren, Germany) according to the manufacturer instructions using the TECAN Freedom EVO System (TECAN, Männedorf, 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 [57]. 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 the observation period, all surviving birds were euthanized by Isoflurane® (CP-Pharma, Burgdorf, 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.

4.8. Histopathology and Immunohistochemistry

The severity of pathohistological lesions and distribution of recombinant viruses in the 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 H5N1_HA4_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 [58,59]. The distribution of NP antigen in the endothelium and parenchyma was blind semiquantitatively scored on an ordinal 0 to 3 scale: 0 = negative; 1 = focal or oligofocal, 2 = multifocal, and 3 = coalescing to diffuse immunoreactive cells.

4.9. 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 of <0.05 was considered significant. All analyses were done using GraphPad Prism 8 software (CA, USA).

4.10. Biosafety

All recombinant DNA protocols were approved by the State Office for Health and Social Affairs of Western Pomerania (LAGuS MV-AZ: 6/08-2/96). Gain-of-function experiments were approved by the German Research Foundation (DFG: VE 780/1) and by the biorisk committee of the Friedrich-Loeffler-Institut (FLI) before starting the project. Experiments with HA specifying polybasic cleavage sites were done in the biosafety level (BSL) 3 laboratory and animal facilities at the FLI. All work was done by experienced researchers who participated in the Project Manager Course for Genetic Engineering and followed the regulations for handling genetically modified organisms.

Author Contributions: E.M.A., J.V., E.B.-F. and T.C.M. conceived and designed the study; M.G., D.S., A.H.S. and E.M.A. conducted the animal experiments; B.C. provided the H4N2 virus; R.U. and O.I.F. conducted the histopathological analysis; M.G. conducted the statistical analysis; MG and EBF conducted the in-vitro characterization. E.M.A. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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(III) Impact of Mutations in the Hemagglutinin of H10N7 Viruses Isolated from Seals on Virus Replication in Avian and Human Cells

III

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Impact of Mutations in the Hemagglutinin of H10N7 Viruses Isolated from Seals on Virus Replication in Avian and Human Cells

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Abstract: Wild birds are the reservoir for low-pathogenic avian influenza viruses, which are frequently transmitted to domestic birds and occasionally to mammals. In 2014, an H10N7 virus caused severe mortality in harbor seals in northeastern Europe. Although the hemagglutinin (HA) of this virus was closely related to H10 of avian H10N4 virus, it possessed unique nonsynonymous mutations, particularly in the HA1 subunit in or adjacent to the receptor binding domain and proteolytic cleavage site. Here, the impact of these mutations on virus replication was studied in vitro. Using reverse genetics, an avian H10N4 virus was cloned, and nine recombinant viruses carrying one of eight unique mutations or the complete HA from the seal virus were rescued. Receptor binding affinity, replication in avian and mammalian cell cultures, cell-to-cell spread, and HA cleavability of these recombinant viruses were studied. Results show that wild-type recombinant H10N4 virus has high affinity to avian-type sialic acid receptors and no affinity to mammalian-type receptors. The H10N7 virus exhibits dual receptor binding affinity. Interestingly, Q220L (H10 numbering) in the rim of the receptor binding pocket increased the affinity of the H10N4 virus to mammal-type receptors and completely abolished the affinity to avian-type receptors. No remarkable differences in cell-to-cell spread or HA cleavability were observed. All viruses, including the wild-type H10N7 virus, replicated at higher levels in chicken cells than in human cells. These results indicate that H10N7 acquired adaptive mutations (e.g., Q220L) to enhance replication in mammals and retained replication efficiency in the original avian host.

Keywords: influenza; H10N7; harbor seals; receptor binding; adaptation; poultry; interspecies transmission

1. Introduction

Influenza A virus, a member of the family *Orthomyxoviridae*, possesses an RNA genome of eight gene segments that encodes at least 10 viral proteins and is enclosed in a nucleoprotein wrapped in a lipid bilayer envelope [1]. According to the antigenic properties of two membrane glycoproteins, hemagglutinin (HA) and neuraminidase (NA), 18 HA and 11 NA subtypes are differentiated [2,3]. Each virus displays distinct HA and NA proteins. Except for H17N10 and H18N11, which were isolated from bats [2], all other HA and NA subtypes were isolated from avian species [3]. Wild aquatic birds are the reservoir for all avian influenza viruses (AIVs). Interspecies transmission from wild birds to domestic birds and mammals has been frequently reported [4,5]. It has been estimated that more

than 200 bird species are susceptible to AIVs [4]. Mammals, including humans, mink, horses, pigs, raccoons, and aquatic animals, are accidental hosts for AIVs, producing infections from asymptomatic to lethal [1,6].

The HA of influenza viruses is a major determinant of cross-species transmission, virulence, and immunogenicity [7]. HA consists of two polypeptides, the N-terminal HA1 and the C-terminal HA2, that remain connected by a disulfide bridge after the proteolytic activation of HA0 by host cell proteases acting at the cleavage site motif [8]. HA1 contains the receptor binding domain (RBD), which forms a shallow pocket in the head domain and is surrounded by 130-loop, 150-loop, 190-helix, and 220-loop structures. Some mutations in or adjacent to the RBD may modulate the binding affinity of influenza viruses to sialic acid (SA), linked to the sugar galactose in α2,3 orientation in AIVs, or in α2,6 orientation in human influenza viruses [3,6]. Moreover, HA1 possesses five immunogenic epitopes, designated A to E, which are major regions for stimulation of and binding to antibodies. Epitopes A and B are located in the head region adjacent to the pocket formed by the RBD. Mutations in these epitopes result in antigenic drift, enabling the virus to escape from the host immune response [9,10].

Avian influenza H10Nx viruses or antibodies have been detected in mink, walrus, raccoons, pigs, dogs, and humans [11–17]. In 2014, high mortality of harbor seals (*Phoca vitulina*) in northwestern Europe was associated with infection by H10N7 influenza virus [18–22]. The virus was first reported in seals in Sweden and Denmark, and subsequently spread to seals off the coasts of Germany and the Netherlands [23]. Although the virus had the capacity to replicate in the mammalian respiratory tract, e.g., in ferrets, the observed high mortality was most likely due to secondary bacterial infections [21]. Genetically, the virus was closely related to contemporary viruses isolated from wild and domestic birds in Europe. In particular, HA showed 98–99% identity to A/mallard/Sweden/133546/ 2011(H10N4) [18–20]. Although several unique amino acid substitutions in the HA protein of seal viruses compared to putative avian parental strains have been described [18–20], the biological function of these mutations, including increased affinity to mammalian receptors, remains unknown. In this study, we compared the HA protein of H10N7 viruses isolated from seals and birds from Europe. Mutations unique to the seal virus in the HA1 domain were identified and their impact on receptor binding specificity, virus replication in avian and mammalian cells, cell-to-cell spread, and cleavage activation was investigated.

2. Materials and Methods

2.1. Viruses and Cells

A/seal/Germany/AR2351/1/14(H10N7) was obtained from the virus repository at the Friedrich-Loeffler-Institut and kindly provided by Timm C. Harder. A/turkey/England/384/79(H10N4) was kindly provided by Ian Brown at the Animal and Plant Health Agency, Weybridge, UK. A/PR/8/1934(H1N1) was kindly provided by J. Stech, FLI, and quail H4N2 virus was kindly provided by Beate Crossley, University of California, Davis, CA, USA. Human-embryonic kidney 293T cells (HEK-293T), Madin-Darby canine kidney cells type II (MDCKII), and human lung adenocarcinoma 549 cells (A549) were obtained from the cell-culture collection at FLI. Primary chicken embryo kidney (CEK) cells were prepared according to standard procedures [24].

2.2. Sequence Analysis

Sequences of the hemagglutinin of H10N7 viruses isolated from harbor seals in Europe in 2014 and their avian H10Nx counterparts, in addition to those detected in mammals, as well as avian H10Nx viruses from non-European countries were retrieved from the Global Initiative on Sharing Avian Influenza Data (GISAID) and the Influenza Virus Database of the National Center for Biotechnology Information. Acknowledgment of authors and laboratories submitting to the GISAID is provided in Supplementary Table S1. All sequences were aligned with Multiple Alignment using Fast Fourier Transform [25], then visualized and edited by BioEdit 7.1.7 (Ibis Therapeutics, Carlsbad, CA, USA) [26].

Asparagine-linked potential glycosylation was predicted by the motif N-X-S/T, where X can be any amino acid (aa) except proline. The H10-HA numbering in this study excludes the 16-residue signal peptide. Predicted locations of the HA mutations mentioned in this study were imposed on the tertiary structure of the HA protein of H10N4 virus using SWISS-MODEL (http://swissmodel.expasy.org/) and then viewed in Geneious v.8.1.3 (Biomatters Ltd., Auckland, Australia) and edited manually.

2.3. Generation of Recombinant Viruses

cDNA of all 8 segments of H10N4 virus and the HA segment of H10N7 virus was generated using a universal primer targeting the conserved 12 nucleotides at the 5'-end as previously published [27]. Each gene segment was amplified using Phusion PCR (New England BioLabs, Frankfurt am Main, Germany) and segment-specific primers (Eurofins, Ebersberg, Germany) [28]. Amplicons were excised and extracted from agarose gels using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Purified products were cloned in pHWS*ccdB* [28], followed by transformation of competent *E. coli* strain TOP10™ (Invitrogen, Thermo Fisher Scientific, Schwerte, Germany), XL1-Blue™, or SURE2™ (Stratagene Europe, Amsterdam, Netherlands). Plasmids were extracted by Qiagen Plasmid Mini, Midi, or Maxi Kit (Qiagen, Hilden, Germany). DNA concentration was adjusted to about 1 μg/μL. Insertion of indicated mutations in the HA of H10N4 was done by QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Waldbronn, Germany). Primers used for generation of mutants are available upon request. Sequences were analyzed to exclude any unwanted mutation by Sanger sequencing using an ABI BigDye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems, Langen, Germany).

All recombinant viruses were rescued after transfection of mixed HEK293T and MDCKII cell culture using Lipofectamine® 2000 and Optimum [28]. Viruses were propagated in 9-to-11-day-old specific pathogen-free embryonated chicken eggs. Inoculated eggs were candled daily for survival of embryos. Eggs that contained dead embryos and those that survived for 5 days post-inoculation were chilled at 4 °C before harvesting of the allantoic fluid. Hemagglutination test was done using 1% chicken erythrocytes, and hemagglutinating units were determined as described [29]. Allantoic fluids with an HA titer $>2^4$ and bacteria-free as determined on blood agar plates were pooled, aliquoted, and stored at −70 °C. Infectivity titers were determined by plaque assay as described below.

2.4. Replication Kinetics

Replication kinetics of recombinant viruses were compared on A549 and CEK cells using 1 plaque-forming unit (PFU) per 1000 cells for 1, 8, 24, and 48 h postinfection (hpi). The cells were infected in the presence of 2 μg/mL trypsin and then incubated at 37 °C or 33 °C with 5% CO_2. At the indicated time points, cells and supernatants were harvested and stored in cryotubes at −80 °C until use. Virus titers were quantified by plaque assay using MDCKII cells. The assay was conducted in duplicate and repeated 2 to 3 times, and the results are expressed as average and standard deviation of all replicates.

2.5. Plaque Test and Cell-to-Cell Spread

Virus was titrated using MDCKII cells in the presence of trypsin using 10-fold serial dilutions in minimum essential medium (MEM). Virus dilutions were added to the cells for 1 h at 37 °C and 5% CO_2, and then the inocula were removed by absorption of infected medium by vacuum. Cells were washed with 1× PBS (pH 7.4) and then overlaid with semisolid agar containing MEM supplemented with bovine serum albumin (BSA). All plates were incubated at 37 °C and 5% CO_2 for 3 days, then fixed by formaldehyde containing crystal violet for at least 1 day. Virus titers were expressed as plaque-forming unit per ml (PFU/mL). To investigate the impact of specific mutations on cell-to-cell spread in MDCKII cells in the presence of trypsin, 50 to 100 plaques were measured using Nikon Instruments NIS Elements Basic Research software (version 4.0, Nikon, Duesseldorf, Germany). Results are shown as percentage relative to plaques produced by the wild-type H10N4 virus.

2.6. Receptor Binding Specificity Assay

Avian α2,3-SA specificity was determined by solid-phase binding assay [30,31]. Briefly, asialofetuin–horseradish peroxidase (HRP) conjugate was sialylated using CMP-sialic acid (Sigma Aldrich, Steinheim, Germany) and α-2,3-(*N*)-sialyltransferase from *Pasteurella multocida* (Sigma Aldrich, Germany). Twelve well plates were coated with 10 µg/mL fetuin from fetal bovine serum (Sigma Aldrich, Germany). Viruses were adjusted to 5 $\log_2$ HA units and 50 µL of indicated viruses was added, followed by incubation of plates overnight at 4 °C. Unbound virus was removed by aspiration, and the plates were washed with PBS and blocked by 0.2 mL of PBS containing 2% bovine serum albumin (Sigma Aldrich, Germany) for 1 h at room temperature. Plates were washed with PBS containing TWEEN®80 (Sigma Aldrich, Germany). Twofold dilution of 50 µL α2,3-labeled fetuin-HRP was done, and the solution was incubated for 1 h at 4 °C then washed with PBS containing TWEEN®80. A total of 100 µL of tetramethylbenzidine substrate was added for 30 min at room temperature, and the reaction was stopped with 50 µL of 50 mM H_2SO_4. The optical density was measured at 450 nm using a Tecan ELISA Reader (Tecan, Crailsheim, Germany).

Receptor binding specificity to mammalian α2,6-linked SA was tested using modified turkey erythrocytes [32]. Briefly, SA was removed by incubation of 1% turkey erythrocytes (TRBCs) with Vibrio cholerae neuraminidase (Sigma Aldrich, Germany) [32]. Desialylated Turkey erythrocytes (TRBCs) were suspended in PBS containing 1% BSA. Loss of TRBC hemagglutination activity was confirmed by incubation with human H1N1/PR8 with high affinity to human-like receptors and avian H4N2 with high affinity to avian receptors. TRBCs were resialylated using α2,6-(*N*)-sialyltransferase (Takara ClonTech, Saint-Germain-en-Laye, France) in a final concentration of 1.5 mM CMP-sialic acid (Sigma-Aldrich, Germany). Modified TRBCs were suspended in PBS containing 1% bovine serum albumin to a final concentration of 0.5%. Resialylation was confirmed by hemagglutination using PR8 and H4N2 viruses. The affinity of all recombinant viruses (adjusted to ~10^7 PFU/mL) to different receptors was compared using standard hemagglutination assay against modified TRBCs, desialylated RBCs, and original turkey RBCs according to the World Organization for Animal Health (OIE) protocol [29]. The assay was run in duplicate and repeated twice. Results are expressed as average of all replicates.

2.7. Heat Stability

The stability of indicated viruses was tested using 600 µL aliquots containing 10^5 PFU of each virus after incubation at 56 °C for different time durations (0, 0.5, 1, 2, 3, and 4 h). The test was conducted in duplicate. The reduction in virus infectivity was determined by plaque assay.

2.8. Western Blot Analysis

MDCKII cells were seeded in T25 flasks 1 day before infection. The cells were washed with MEM with 5% bovine serum albumin (BSA) and then infected with virus at a multiplicity of infection of >1 PFU per cell at 37 °C for 1 h. Cells were washed twice with PBS, and MEM with BSA was added with or without trypsin (2 µg/mL) at 37 °C/5% CO_2. At 6 and 24 hpi, the cells were harvested with cell scrapers and collected in Falcon tubes. The cell suspension was centrifuged at 14,000 rpm for 15 min. The supernatant was aspirated and the cell pellets were washed with PBS. This was repeated twice before suspending cell pellets in Laemmli buffer (Sigma-Aldrich, Germany) and PBS at a ratio of 1:1. Moreover, deglycosylation of indicated viruses was studied using PNGase F (New England BioLabs, Frankfurt am Main, Germany) at 37 °C for 1 h following the vendor's recommended protocol. All samples were stored at −20 °C until further analysis. Samples were thawed at room temperature, then incubated at 99 °C for 5 min, followed by centrifugation at 14,000 rpm for 5 min. The proteins were separated on 10% or 12% SDS-PAGE along with BenchMark™ Pre-Stained Protein Ladder in SDS-PAGE buffer for 45 min at 200 volts. For the step of transferring the proteins onto a nitrocellulose membrane, a semidry blotting gadget was used at 25 volts for 2 h. Transferring viral proteins to

nitrocellulose membranes and processing the blots were done as previously described [33], with little modification. The serum used was obtained 10 days after infection of chickens with H10N4 virus intravenously in a previous experiment. Serum in 0.5% skim milk (1:100) was added and incubated at 37 °C overnight. Then the membrane was washed twice with Tris-Buffered Saline containing Tween (TBST) for 30 s, once for 15 min, then twice for 5 min. Thereafter, blots were incubated with peroxidase-conjugated species-specific secondary antibodies at a dilution of 1:20,000 in TBST for 30 min, followed by several washing steps and continuous shaking. Immunodetection was achieved by chemiluminescence using Supersignal West Pico chemiluminescent substrate kit (Pierce, Thermo Scientific, Rockford, IL, USA). Images were captured using a Bio-Rad VersaDoc imaging system (Bio-Rad, Hercules, CA, USA) and Quantity One software (version 4.6.3, Bio-Rad, Hercules, CA, USA).

2.9. Statistics

Variations in replication kinetics in different cells, heat stability, and receptor binding affinity were assessed using ANOVA with post hoc Tukey test. Differences in plaque size were compared using Kruskal-Wallis test and Wilcoxon test with Bonferroni correction. Data were analyzed using R version 2.14.0 from the R Foundation for Statistical Computing, available at the R-project website (http://www.r-project.org), and differences were considered significant at a p-value < 0.05.

3. Results

3.1. Sequence Analysis

Sequences of all European H10Nx viruses from birds and mammals in GenBank and GISAID available to 12, October, 2017 were retrieved and analyzed. A total of 26 seal virus sequences were collected, from Sweden (n = 4), Denmark (n = 5), Germany (n = 11), and the Netherlands (n = 6), isolated in 2014 (n = 25) and 2015 (n = 1). In addition, 124 sequences of H10N1 to H10N9 viruses from domestic and wild birds isolated from 1949 to 2015 were deposited in the databases. Amino acid differences in the HA1 of seal viruses compared to the avian counterparts studied herein are summarized in Table 1. Nine mutations—E82K, S113N, T165A, Q204K, N206S, Q220L, N236K, T238I, and M321V (H10 numbering corresponding to positions 91, 122, 171, 210, 212, 226, 242, 244, and 327 in H3 numbering)—were common in the HA1 of seal viruses, with a prevalence rate ranging from 57.7 to 100%. These positions are highly conserved in the European avian viruses, ranging from 84.7 to 100% (Table 1). None of these mutations was observed in the available sequences from mink (n = 2), pigs (n = 1), or humans (n = 4) (Supplementary Table S2). Except for M321V, the prevalence of these mutations in non-European H10Nx viruses ranged from 0 to 5.7% (Supplementary Table S2). All mutations except E82K and M321V reside in the HA head domain (Figure 1). E82K is located in the proximity of the stalk domain (adjacent to the head domain), and M321V is a part of the cleavage site. Twenty-five out of 26 seal viruses have ^{318}PE(L/I)VQGR^{324} | GLFGAIA (the cleavage site is after R324 and upstream of the GLFGAIA peptide) as the cleavage site motif, whereas avian H10Nx viruses possess the cleavage site motif ^{318}PE(L/I/V)MQGR^{324} | GLFGAIA. Mutations in position N236K and/or T238I resulted in absence of glycosylation at ^{236}NIT^{238} (Figure 2A) due to lysine and/or isoleucine substitution ^{236}KIT^{238} or ^{236}KII^{238}. The molecular weight of glycosylated wild-type H10N4 HA is higher than that of HA of N236K and T238I carrying viruses (Figure 2A). This *N*-glycosylation motif is highly conserved in avian viruses, where 118 out of 124 (95.9%) sequences contain this potential glycosylation site. On the monomer structure of HA, Q220L resides in the inner rim of the receptor binding pocket, whereas Q204K, N206S, N236K, and T238I are located parallel to each other near the 190-helix and 220-loop (Figure 1).

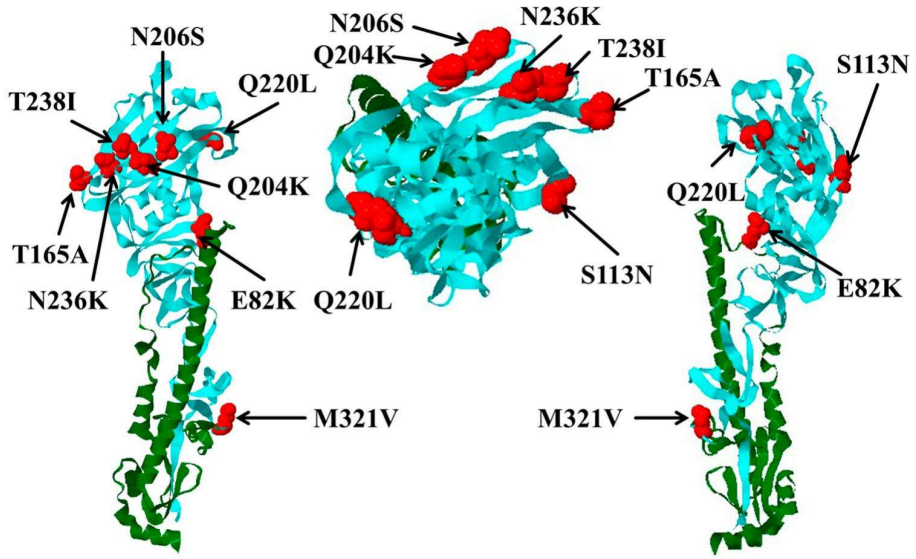

Figure 1. Locations of hemagglutinin 1 (HA1) mutations and their impact on receptor binding activity and cleavability of hemagglutinin. Predicted locations of HA mutations in seal H10N7 viruses compared to avian H10Nx viruses are shown in red. HA1 is shown in cyan and HA2 in green. Left and right views are about 180 degrees apart (e.g., front vs. back views), and head view is in the middle.

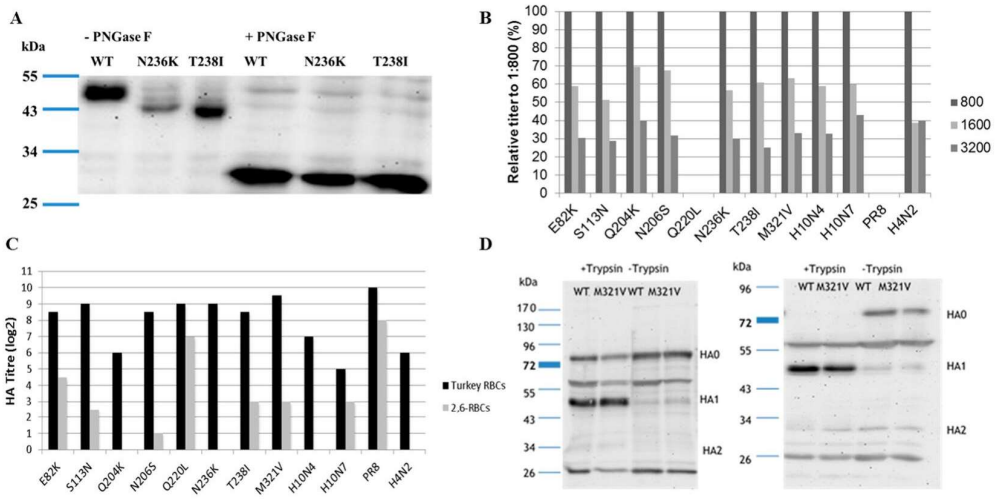

Figure 2. (**A**) Molecular weight of the HA of H10N4 (wild type) compared to viruses carrying N236K or T238I without (-PNGase) or with (+PNGase) treatment using PNGase F; (**B**) affinity of different recombinant H10 viruses to avian-type receptors using different concentrations (1:800; 1:1600, and 1:3200) of 2,3-labelled fetuin analyzed in solid-phase assay; (**C**) receptor binding affinity to turkey erythrocytes expressing both avian and mammalian sialic acid receptors, and modified turkey erythrocytes expressing 2,6-mammalian receptors only. Results are expressed as average of hemagglutination titer of two independent runs, each run in duplicate; (**D**) cleavage of HA of H10N4 compared to H10N4 with M321V substitution in the presence (+Trypsin) or absence (-Trypsin) of trypsin 6 or 24 h after infection of Madin-Darby canine kidney cells type II (MDCKII) using polyclonal antiserum generated 10 days after intravenous inoculation of chickens with H10N4 virus. All viruses were constructed by reverse genetics, except H10N7 wild-type virus, which was used as a control.

Table 1. Prevalence of fixed mutations in seal viruses compared to other European H10Nx viruses.

Mutation		Avian Viruses	Number n = 124 (%)	Seal Viruses	Number n = 26 (%)	Substitution
H10 Numbering	H3 Numbering					
82	91	E	116 (93.5)	K	25 (96.2)	E82K
113	122	S	119 (96.0)	N	25 (96.2)	S113N
165 *	171	T	124 (100.0)	A	22 (84.6)	T165A
204	210	Q	124 (100.0)	K	22 (84.6)	Q204K
206	212	N	123 (99.2)	S	15 (57.7)	N206S
220	226	Q	124 (100)	L	17 (65.4)	Q220L
236 **	242	N	118 (95.9)	K	23 (88.5)	N236K
238 **	244	T	123 (99.2)	I	15 (57.7)	T238I
321 ***	327	M	105 (84.7)	V	25 (96.2)	M321V

* Recombinant virus with this mutation could not be rescued. ** Mutations in these positions resulted in deglycosylation of ^{236}NIT^{238} to ^{236}KIT^{238} or ^{236}KII^{238}. *** Mutation in this position changed the cleavage site from ^{318}PE(L/I)MQGR^{324} | GLFGAIA to ^{318}PE(L/I)VQGR^{324} | GLFGAIA.

3.2. Rescue of Recombinant Viruses

The wild-type H10N4, H10N4 carrying HA from seal H10N7, and single mutants carrying unique mutations in positions E82K, S113N, Q204K, N206S, Q220L, N236K, T238I, or M321V, except T165A, were successfully rescued. All viruses were propagated in specific pathogen-free eggs, and the plaque titer was determined as described above.

3.3. Receptor Binding

Affinity to avian and mammalian SA receptors was compared using modified TRBCs and solid phase assay. Avian influenza viruses are able to bind to SA with α2,3 (avian-like) and α2,6 (mammalian-like) linkages. Therefore, the viruses were tested in an HA test with TRBCs, which carry both α2,3- and α2,6-SA, and modified TRBCs, which carry only the α2,6 human-type receptors. Results indicate that H10N4 virus recognized only the avian receptors and did not bind to the modified TRBCs. The seal H10N7 virus exhibited dual receptor affinity to both avian and mammalian receptors in TRBCs (Figure 2B,C). Q220L, the mutation at RBD, remarkably increased the affinity to mammalian-type receptors and completely abolished binding of H10N4 to avian-type receptors, thus making it similar to the human H1N1/PR8 (Figure 2C). Also, E82K and, to a lesser extent, T238I, M321V, S113N, and N206S increased the affinity of H10N4 to mammalian-type receptors (Figure 2C). Other viruses showed comparable affinity to the avian receptors.

3.4. Western Blot

Since M321V is very close to the HA cleavage site, the impact on cleavability was assessed by Western blot in MDCKII cells after single (6 hpi) or multiple (24 hpi) cycles with or without the addition of exogenous trypsin (Figure 2D). In the absence of trypsin, both viruses, the reverse-engineered H10N4 and H10N4 carrying M321V, were inefficiently cleaved (Figure 2D). Conversely, in the presence of trypsin, the HA of both viruses was cleaved into HA1 and HA2 subunits without obvious difference (Figure 2D).

3.5. Replication Kinetics

Viruses replicated in avian and mammalian cell lines. The temperature in the upper respiratory tract of birds is about 39 °C, which is higher than the temperature of the respiratory tract of mammals (about 33 °C). Therefore, replication kinetics of different mutants were studied in CEK at 37 °C and in A549 cells at 37 °C and 33 °C. All viruses replicated in CEK (Figure 3A) to at least 10-fold higher titers than in A549 at 37 °C (Figure 3B).

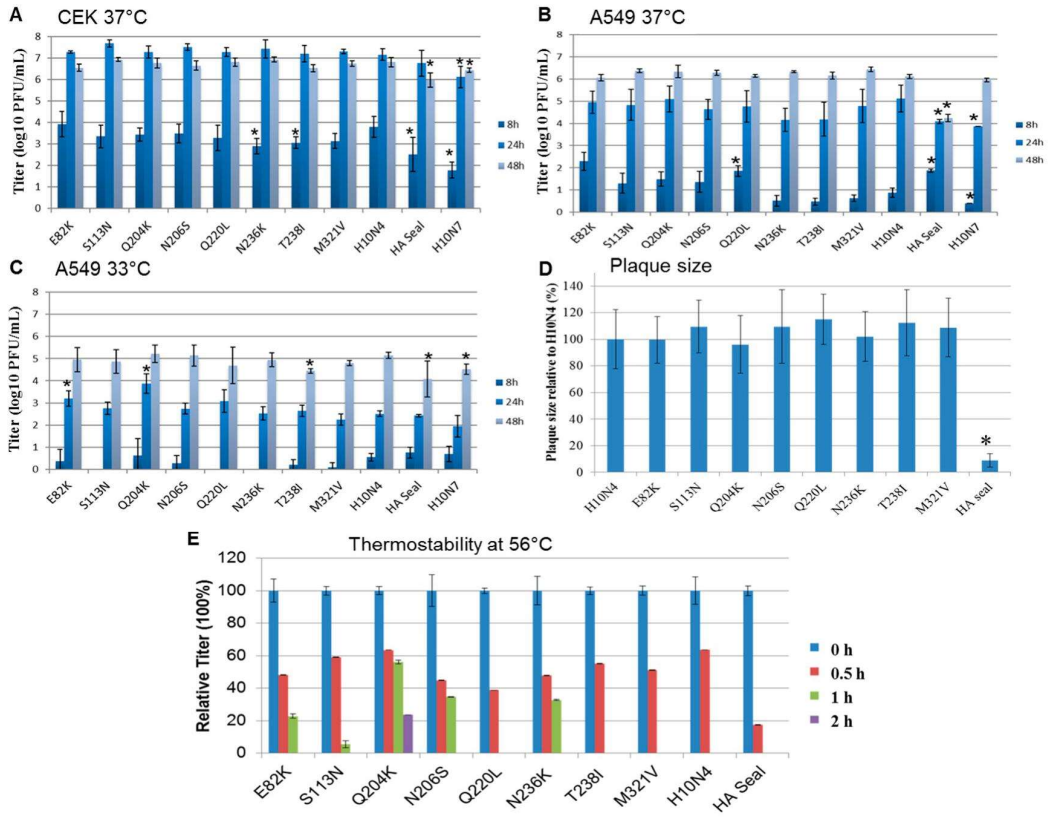

Figure 3. Replication kinetics and cell-to-cell spread of recombinant H10 viruses. Shown are the averages and standard deviations of replication kinetics of indicated viruses in (**A**) primary chicken embryo kidney cells at 37 °C and (**B**) human lung adenocarcinoma cells at 37 °C and (**C**) 33 °C at 8, 24, and 48 h postinfection. Cell-to-cell spread was assayed by measuring 50 to 100 plaques in MDCKII cells; (**D**) results are expressed as percentage of the average diameter of plaques induced by H10N4. Statistically significant values compared to the H10N4 virus are shown by asterisks. Thermostability of 10^5 plaque-forming units (PFU) of each virus was determined after incubation at 56 °C for 4 h. The test was conducted in duplicate and repeated twice, and the virus titer was determined by plaque assay in MDCKII cells. All viruses were inactivated after 2 h; (**E**) shown are the relative titers at each indicated time point compared to titers before inactivation.

In CEK cells, mutations in HA1 did not significantly affect H10N4 virus replication at 24 and 48 h (Figure 3A). Replication of H10N7 and the recombinant expressing HA seal was significantly lower than that of the parental H10N4 ($p < 0.002$ and 0.03, respectively), where the titers of the seal virus H10N7 were 10- to 100-fold lower than those of the avian H10N4 virus. Also, at 8 hpi, N236K and T238I replicated at significantly lower levels than H10N4 virus ($p < 0.004$) (Figure 3A).

In A549 cells at 37 °C, at 8 hpi, H10N4 viruses carrying E82K, Q220L, or H10 from seal replicated to significantly higher levels than the H10N4 virus ($p < 0.0008$) and, interestingly, about 100 times higher than the seal H10N7 ($p < 0.003$) (Figure 3B). However, at 24 hpi, mutations did not affect the replication efficiency of H10N4 virus. At 24 hpi, HA-seal H10N4 virus and seal H10N7 virus replicated at significantly lower levels compared to the H10N4 virus ($p < 0.02$ and 0.007, respectively). At 48 hpi, all viruses except seal-HA H10N4 virus reached comparable replication titers (Figure 3B).

In A549 cells at 33 °C, replication of all viruses was reduced compared to their growth rate at 37 °C (Figure 3C). At 8 hpi, the titers were very low for all viruses and below the detection limit for mutants S113N, Q220L, and N236K (Figure 3C). Remarkably, E82K and Q204K significantly increased replication of H10N4 virus at 24 hpi ($p < 0.002$). All viruses replicated to approximately comparable

levels at 48 hpi, where H10N4-T238I, HA seal, and H10N7 replicated at significantly lower titers than H10N4 ($p < 0.04$) (Figure 3C).

3.6. Cell-to-Cell Spread

To determine cell-to-cell spread, plaque assays using MDCKII cells were performed, and plaque sizes were measured and compared to wild-type H10N4. Results indicate that single mutations in HA1 did not significantly affect cell-to-cell spread. However, H10N4 virus carrying HA from seal virus produced very small plaques compared to other viruses ($p < 0.00001$) (Figure 3D). The seal H10N7 virus produced minute plaques, which could only be visualized under a microscope.

3.7. Heat Stability

Stability of AIV at high ambient temperature may facilitate the persistence or spread of the virus. Thus, it was important to investigate the impact of those unique mutations on heat stability. After 30 min at 56 °C, the titers of recombinant H10N4 viruses were reduced to 18 to 64% of their original titers (Figure 3E). Virus specifying H10 HA from seal was rapidly inactivated, while H10N4 viruses containing mutations N204K, N206S, and N236K and, to a lesser extent, E82K and S113N were infectious for at least 1 h. Only recombinant H10N4 virus with the N204K mutation retained infectivity for at least 2 h (Figure 3E). All viruses were inactivated after 2 h.

4. Discussion

While the mutation rate of AIVs in wild aquatic birds is low and considered to be mainly in a "stasis" form [1], interspecies transmission from wild birds to domestic birds or to mammals requires adaptive genetic changes to facilitate virus replication in these non-reservoir hosts [34]. Changes in receptor binding from avian to mammalian receptors are essential for efficient interspecies transmission in mammals or the emergence of pandemic viruses [35]. Seals may act as mixing vessels for the generation of pandemic viruses due to frequent infection by human and avian viruses [22,36–40]. Although controversial, some studies have shown that the respiratory tract of seals possesses both avian-like and human-like receptors [37], while others have confirmed only the presence of avian 2,3-SA in the lungs of seals [41]. In 2014, H10N7 viruses were isolated from seals on the northeastern coast of Europe during one of the largest outbreaks of AIV in sea mammals. All gene segments of this virus were closely related to avian influenza viruses, including H10N4, isolated from wild birds in Europe, suggesting direct transmission of one avian influenza virus to seals [18,19,22]. However, compared to the avian H10 viruses, the seal viruses carried unique mutations in HA1, mostly in the head region or close to the receptor binding domain. This could be due to the natural selection pressure of host species, which acts first on surface proteins [18]. Using reverse genetics, we successfully generated recombinant H10N4 viruses carrying full seal H10 or single mutations specific to the HA1 of seal H10N7 viruses isolated in the 2014 outbreak.

In contrast to avian H10N4 and H4N2 viruses, seal H10N7 virus bound to mammalian SA receptors but retained binding affinity to avian-type receptors. These results are also in accordance with previous reports, which described dual receptor affinity of avian H3N8 viruses isolated from New England harbor seals in 2011, in which the H3N8 affinity to avian 2,3-SA receptors was significantly higher than binding to 2,6-SA receptors [36,42–44]. Increased binding affinity to 2,6-SA receptors may be attributed to the Q220L mutation. This position resides directly in the RBD. H10N4 virus carrying Q220L bound effectively to 2,6-SA receptors, as did human H1N1 virus. It was previously described that double mutations Q226L and G228S (H3 numbering, which corresponds to 220 and 222 in H10 viruses) are essential for the adaptation of avian H2N2 and H3N2 subtypes in humans [45]. All seal viruses carry the avian G222 residue. Also, L220 has been implicated in the adaptation of equine H3N8 virus in dogs [46]. Therefore, this Q220L mutation that is present in a major fraction of seal H10N7 viruses may have been similarly important in their stepwise adaptation in seals. Nevertheless, all H10 viruses in this study replicated at lower levels in human lung cells than in avian cells, which

may indicate poor adaptation to human cells. However, viruses carrying E82K, Q220L (with high affinity to 2,6-SA receptors), and HA from seal H10N7 virus produced relatively high titers in A549 cells after 8 h at 37 °C. Remarkably, N204K, N206S, and N236K and, to lesser extent, E82K and S113N increased thermostability of the virus, which may be important for efficient transmission between seals. It has been shown that some phenotypic traits of HA, such as human receptor binding preference and heat stability, are critical for airborne transmission of H5N1 AIV between ferrets [47,48]. Interestingly, the lack of glycosylation at the ^{236}NIT^{238} motif due to mutations in position N236K and/or T238I increased affinity to human-type receptors and reduced H10N4 virus replication in CEK, suggesting a role in virus adaptation, as seen in several human H5N1 viruses [49,50]. The impact of these mutations in double, triple, or multiple combinations remains to be investigated. Beyond HA, other mutations, particularly in polymerase genes, may be additionally required for efficient replication in human cells [51]. It was also observed that both the seal isolate H10N7 and the "seal-HA H10N4" replicated at lower levels than recombinant avian H10N4 viruses in CEK cells, which may indicate a gradual loss of fitness for the original avian host. Moreover, in previous reports, mutations in the vicinity of the cleavage site of AIV affected cleavage activation of the virus and were correlated with increased virulence [52,53]. The seal H10N7 virus possesses M321V, which is located in the vicinity of the HA cleavage site. However, we could not observe any obvious difference in the cleavability of HA into HA1 and HA2 subunits in the presence or absence of trypsin. Whether this mutation affects the cleavability of the virus by other proteases (e.g., TMPRSS2, HAT) [54] remains to be investigated.

Altogether, although seal H10N7 virus retained strong binding affinity to avian-type receptors, the virus showed increased affinity to mammalian-type receptors, mainly due to the Q220L mutation in the receptor binding domain. The mutations analyzed appear to contribute to virus adaptation in seals/mammals and to a virus that can replicate and infect both avian and mammalian cells.

Supplementary Materials: The following are available online at www.mdpi.com/1999-4915/10/2/83/s1, Table S1: Acknowledgment of authors and originating and submitting laboratories of the sequences from GISAID's EpiFlu database, Table S2: Prevalence of different mutations in mammalian and non-European H10Nx viruses.

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Author Contributions: Elsayed M. Abdelwhab and Thomas C. Mettenleiter conceived and designed the experiments; Anne Dittrich, David Scheibner, Ahmed H. Salaheldin, Marcel Gischke, and Elsayed M. Abdelwhab performed the experiments; Elsayed M. Abdelwhab analyzed the data; Elsayed M. Abdelwhab, Jutta Veits, and Thomas C. Mettenleiter wrote the paper.

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Supplemental Material

TAB]

We acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFlu™ Database on which this research is based. The list is detailed below.

All submitters of data may be contacted directly via the GISAID website ***www.gisaid.org***

Segment ID	Segment	Country	Collection date	Isolate name	Originating Lab	Submitting Lab	Authors
EPI178459	HA	Italy	1967-Jan-01	A/Turkey/Italy/928/1967		Istituto Zooprofilattico Sperimentale della Lor	
EPI169461	HA	United Kingdc	1985-Jan-01	A/Fowl/Hampshire/PD378/1985	Veterinary Laboratories Agency, Weybridge	National Veterinary Institute	
EPI178543	HA	Italy	1966-Jan-01	A/Quail/Italy/1966		Istituto Zooprofilattico Sperimentale della Lor	
EPI278812	HA	Belgium	2009-Mar-21	A/Tadorna tadorna/Belgium/3441-P2/2009	Veterinary and Agrochemical Research Institute	Veterinary and Agrochemical Research Instit	
EPI372496	HA	Ukraine	2006-May-01	A/pied avocet/Ukraine/05818-NAMRU3/2006	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elass
EPI169477	HA	Germany	1949-Jan-01	A/chicken/Germany/N/1949		National Veterinary Institute	
EPI774514	HA	Netherlands	2015-Apr-29	A/chicken/Netherlands/15007212/15	Central Veterinary Institute	Central Veterinary Institute	Bergervoet, Saskia; Heutink, Rene; Verschuren-Pritz, Sylvia; Harders, Frank; Bossers, Alex; Beere
EPI317614	HA	Germany	2007-Jan-01	A/Mallard/Germany/R2075/2007	Friedrich-Loeffler-Institut	Friedrich-Loeffler-Institut	Stech,O; Weber,S; Mettenleiter,TC; Stech,J
EPI178536	HA	Italy	2007-Jan-01	A/Duck/Italy/60772/2007		Istituto Zooprofilattico Sperimentale della Lor	
EPI178528	HA	Italy	2006-Jan-01	A/Duck/Italy/62330/2006		Istituto Zooprofilattico Sperimentale della Lor	
EPI178493	HA	Italy	2004-Jan-01	A/Duck/Italy/268302/2004		Istituto Zooprofilattico Sperimentale della Lor	
EPI174777	HA	Italy	2006-Jan-01	A/duck/Italy/73383/2006		Istituto Zooprofilattico Sperimentale della Lor	
EPI169500	HA	Belgium	1978-Jan-01	A/duck/Belgium/380/1978		Veterinary and Agrochemical Research Instit	
EPI541472	HA	Denmark	2011-Nov-14	A/mallard/Denmark/16109-4/2011-11-14	Technical University of Denmark	Technical University of Denmark	Hjulsager, Charlotte; Breum, Solvej; Trebbien, Ramona; Larsen, Lars E
EPI552756	HA	Netherlands	2012-Jan-14	A/mallard/Netherlands/1/2012	Erasmus Medical Center	Erasmus Medical Center	Bodewes, Rogier; Bestebroer, Theo M.; Van der Vries, Erhard; Verhagen, Josanne H.; Koopmans,
EPI552753	HA	Netherlands	2010-Nov-26	A/mallard/Netherlands/47/2010	Erasmus Medical Center	Erasmus Medical Center	Bodewes, Rogier; Bestebroer, Theo M.; Van der Vries, Erhard; Verhagen, Josanne H.; Koopmans,
EPI552752	HA	Netherlands	2010-Dec-03	A/mallard/Netherlands/50/2010	Erasmus Medical Center	Erasmus Medical Center	Bodewes, Rogier; Bestebroer, Theo M.; Van der Vries, Erhard; Verhagen, Josanne H.; Koopmans,
EPI552751	HA	Netherlands	2014-Feb-17	A/mallard/Netherlands/1/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes, Rogier; Bestebroer, Theo M.; Van der Vries, Erhard; Verhagen, Josanne H.; Koopmans,
EPI511811	HA	Italy	2007-Jan-01	A/mallard/Italy/4518/2007	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Ve	
EPI169485	HA	United Kingdc	1985-Jan-01	A/Mallard/Gloucestershire/PD374/1985	Veterinary Laboratories Agency, Weybridge	National Veterinary Institute	
EPI332944	HA	France	2009-May-20	A/Anas_platyrhynchos/Camargue/091863/09	Station Biologique "Tour du Valat"	Institut Pasteur	VITTECOQ Marion, GRANDHOMME Viviane
EPI178551	HA	Italy	2006-Jan-01	A/Mallard/Italy/46341-12/2006		Istituto Zooprofilattico Sperimentale della Lor	
EPI278626	HA	Belgium	2009-Apr-01	A/Anas platyrhynchos/Belgium/4455-cls2/2009	Veterinary and Agrochemical Research Institute	Veterinary and Agrochemical Research Instit	
EPI223161	HA	Belgium	2009-Apr-01	A/Anas platyrhynchos/Belgium/4486/2009	Veterinary and Agrochemical Research Institute	Veterinary and Agrochemical Research Instit	
EPI223136	HA	Belgium	2009-Sep-21	A/Anas platyrhynchos/Belgium/09-2249/2008		Veterinary and Agrochemical Research Instit	
EPI541474	HA	Denmark	2014-Jul-01	A/harbor seal/Denmark/14-5061-1lu/2014-07	Technical University of Denmark	Technical University of Denmark	Krog, Jesper Schak; Hjulsager, Charlotte; Larsen, Lars E
EPI709090	HA	Sweden	2014-Sep-07	A/Seal/Sweden/SVA1412040224-SZ5634/H10N7/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709146	HA	Denmark	2014-Sep-30	A/harbor seal/DK/14-10580_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709145	HA	Denmark	2014-Sep-29	A/harbor seal/DK/14-10581_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709144	HA	Denmark	2014-Sep-26	A/harbor seal/DK/14-10585_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709143	HA	Denmark	2014-Aug-29	A/harbor seal/DK/14-8148_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709152	HA	Germany	2014-Nov-03	A/harbor seal/GER/FLI 2508_14/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709151	HA	Germany	2014-Nov-04	A/harbor seal/GER/FLI 2509_14/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709150	HA	Germany	2014-Nov-02	A/harbor seal/GER/FLI 2511_14/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709149	HA	Germany	2014-Nov-05	A/harbor seal/GER/FLI 2512_14/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709148	HA	Germany	2014-Nov-17	A/harbor seal/GER/FLI 2513_14/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709147	HA	Germany	2014-Oct-14	A/harbor seal/GER/PV20756_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709142	HA	Germany	2014-Oct-14	A/harbor seal/GER/PV20756_Tr/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709141	HA	Germany	2014-Oct-22	A/harbor seal/GER/PV20770_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709140	HA	Germany	2014-Oct-22	A/harbor seal/GER/PV20770_Tr/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709139	HA	Germany	2014-Oct-11	A/harbor seal/GER/PV20787_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709138	HA	Germany	2014-Oct-11	A/harbor seal/GER/PV20787_Tr/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709137	HA	Germany	2014-Oct-23	A/harbor seal/GER/PV20982_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709136	HA	Germany	2014-Oct-23	A/harbor seal/GER/PV20982_TrS/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709135	HA	Germany	2014-Dec-23	A/harbor seal/GER/PV20959_NS/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709134	HA	Germany	2014-Oct-07	A/harbor seal/GER/S1032_14_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709133	HA	Germany	2014-Oct-07	A/harbor seal/GER/S1040_14_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709132	HA	Germany	2014-Oct-07	A/harbor seal/GER/S1041_14_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709131	HA	Germany	2014-Oct-07	A/harbor seal/GER/S1042_14_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709130	HA	Germany	2014-Oct-10	A/harbor seal/GER/S1046_14_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709129	HA	Germany	2014-Oct-10	A/harbor seal/GER/S1047_14_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709128	HA	Germany	2014-Oct-07	A/harbor seal/GER/S1048_14_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709127	HA	Germany	2014-Sep-30	A/harbor seal/GER/S1050_14_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709126	HA	Germany	2014-Sep-30	A/harbor seal/GER/S1050_14_TS/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709125	HA	Germany	2014-Oct-01	A/harbor seal/GER/S1052_14_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709124	HA	Germany	2014-Oct-01	A/harbor seal/GER/S1052_14_TS/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709123	HA	Germany	2014-Oct-07	A/harbor seal/GER/S1054_14_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709122	HA	Germany	2014-Oct-07	A/harbor seal/GER/S1054_14_Tr/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709121	HA	Germany	2014-Oct-06	A/harbor seal/GER/S1055_14_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709120	HA	Germany	2014-Oct-06	A/harbor seal/GER/S1055_14_Tr/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709119	HA	Germany	2014-Oct-14	A/harbor seal/GER/S1070_14_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709118	HA	Germany	2014-Oct-14	A/harbor seal/GER/S1070_14_ThS/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709117	HA	Germany	2014-Oct-14	A/harbor seal/GER/S1070_14_Tr/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709116	HA	Germany	2014-Oct-14	A/harbor seal/GER/S1071_14_L/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709115	HA	Germany	2014-Oct-14	A/harbor seal/GER/S1071_14_ThS/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier
EPI709114	HA	Germany	2014-Oct-14	A/harbor seal/GER/S1071_14_TrS/2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes,Rogier

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Supplementary Table S2: Prevalence of different mutations in mammalian and non-European H10Nx viruses

Residue		European Avian H10-viruses	Seal H10N7-viruses	Mammal-viruses (n=7)*		Non-European Avian Viruses (n=842)	
H10-Numbering	H3-Numbering			Avian-like	Seal-like	Avian-like	Seal-like
82	91	E	K	**E: 5(71.4%)**	K: 2 (28.6%)**	**E: 592(70.3%)** D: 244(29%) N: 1 (0.1%)	0
113	122	S	N	**S: 7(100%)**	0	**S: 809(96.1%)** G: 14(1.7%)	N: 13(1.5%)
165	171	T	A	**T: 7(100%)**	0	**T: 834(99%)** I/K: 7(0.9%)	A: 1(0.1%)
204	210	Q	K	**Q: 4(57.1%)** R: 3(42.9%)	0	**E: 779(92.5%)** R: 62(7.4%) H: 1(0.1%)	0
206	212	N	S	**N: 7(100%)**	0	**N: 794(94.3%)**	S: 48(5.7%)
220	226	Q	L	**Q: 7(100%)**	0	**Q: 842(100%)**	0
236	242	N	K	**N: 6(85.7%)** S: 14.3%)	0	**N: 835(99.2%)** S: 6(0.7%) D: 1(0.1%)	0
238	244	T	I	**T: 7(100%)**	0	**T: 841(99.9%)**	I: 1(0.1%)
321	327	M	V	**M: 2(28.6%)** I: 5(71.4%)	0	**M: 154(18.3%)** I: 74(8.8%) A: 45(5.3%) F: 1(0.1%)	V: 568(67.5%)

* Seven available sequences of H10Nx in mammals were retrieved: swine (one H10N5 from China), mink (two H10N4 from Sweden) and humans (four H10N8 from China). Two Australian H10N7 in humans were deposited in the GenBank, however only aa in positions 368 to 532 are available. Therefore, they were not included in the current analysis.

** positive samples were detected in mink.

Written in bold are those sequences with avian-like signatures as found in European H10Nx viruses.

4. Own contribution to publications

Paper I

The role of glycosylation in the N-terminus of the hemagglutinin of a unique H4N2 with a natural polybasic cleavage site in virus fitness in vitro and in vivo

Marcel Gischke, Ola Bagato, Angele Breithaupt, David Scheibner, Claudia Blaurock, Melina Vallbracht, Axel Karger, Beate Crossley, Jutta Veits, Eva Böttcher-Friebertshäuser, Thomas C. Mettenleiter and Elsayed M. Abdelwhab

Virulence 2021, 12, 1; DOI: 10.1080/21505594.2021.1881344

Marcel Gischke:	Sequence analysis; Generation, cloning, mutagenesis and sequencing of expression plasmids coding for gene segments of H4N2; Rescue, propagation and titration of recombinant viruses; Functional characterization of viruses including replication in CEK cells, cell-to-cell spread in MDCKII cells, HA-glycosylation studies with PNGase F and heat stability; Establishment of solid phase binding assay; Main participation in receptor binding studies; Main participation in HA fusion studies; Infection and assessment of ECE; Preparation of chicken embryos; Participation in the animal trial and collection and analysis of samples; Design of the study; Statistical analysis of data; Interpretation of data; Participation in the preparation and correction of the manuscript; Preparation of figures
Ola Bagato:	Virus replication studies in MDCK cells with and without co-expressed HAT or TMPRSS2; Participation in the correction of the manuscript
Angele Breithaupt:	Histopathological and immunohistochemical examination and assessment; Participation in the correction of the manuscript
David Scheibner:	Assessment of clinical status in the animal trial and collection of samples; Participation in the correction of the manuscript

Claudia Blaurock:	Participation in HA fusion studies; Participation in receptor binding studies; Participation in the animal trial and collection of samples; Participation in the correction of the manuscript
Melina Vallbracht:	Establishment of transient transfection based fusion assay; Assistance in HA fusion studies; Participation in the correction of the manuscript
Axel Karger:	Participation in HA-glycosylation studies; Participation in the correction of the manuscript
Beate Crossley:	Isolation, propagation and sharing of A/Quail/California/D113023808/2012 (H4N2); Participation in the correction of the manuscript
Jutta Veits:	Design of the study; Provide funding; Participation in the correction of the manuscript
Eva Böttcher-Friebertshäuser:	Generation and providing MDCK cells with stable HAT or TMPRSS2 expression; Participation in the correction of the manuscript
Thomas C. Mettenleiter:	Design of the study; Provide funding; Participation in the correction of the manuscript
Elsayed M. Abdelwhab:	Design and supervision of the study; Sequence analysis, Planning and implementation of the animal trial; Assessment of clinical status in the animal trial and collection of samples; Interpretation of data; Provide funding; Main participation in the preparation and correction of the manuscript; Corresponding author

Paper II

Insertion of Basic Amino Acids in the Hemagglutinin Cleavage Site of H4N2 Avian Influenza Virus (AIV)-Reduced Virus Fitness in Chickens is Restored by Reassortment with Highly Pathogenic H5N1 AIV

Marcel Gischke, Reiner Ulrich, Olanrewaju I Fatola, David Scheibner, Ahmed H Salaheldin, Beate Crossley, Eva Böttcher-Friebertshäuser, Jutta Veits, Thomas C Mettenleiter and Elsayed M Abdelwhab

IJMS 2020, 21, 7; DOI: 10.3390/ijms21072353

Marcel Gischke:	Generation, cloning, mutagenesis and sequencing of expression plasmids coding for gene segments of H4N2; Rescue, propagation and titration of recombinant viruses; Functional characterization of viruses including replication in CEK an MDCK cells, cell-to-cell spread in MDCK and MDCKII cells, HA-cleavage studies with trypsin; Participation in animal trials and collection and analysis of samples; Participation in section of animals; Design of the study; Statistical analysis of data; Interpretation of data; Participation in the preparation and correction of the manuscript; Preparation of figures
Reiner Ulrich:	Main participation in post-mortem examination of experimental animals; Histopathological and immunohistochemical examination and assessment; Participation in the correction of the manuscript
Olanrewaju I Fatola:	Histopathological and immunohistochemical examination and assessment; Participation in the correction of the manuscript
David Scheibner:	Assessment of clinical status in the animal trials and collection of samples; Main participation in post-mortem examination of experimental animals; Participation in the correction of the manuscript
Ahmed H Salaheldin:	Assessment of clinical status in the animal trial and collection of samples; Participation in the correction of the manuscript

Beate Crossley:	Isolation, propagation and sharing of A/Quail/California/D113023808/2012 (H4N2); Participation in the correction of the manuscript
Eva Böttcher-Friebertshäuser:	Design of the study; Participation in the proteolytic activation using HAT and TMPRSS2, with and without furin inhibitor (MI-1148); Provide funding; Participation in the correction of the manuscript
Jutta Veits:	Design of the study; Provide funding, Participation in the correction of the manuscript
Thomas C Mettenleiter:	Design of the study; Provide funding; Participation in the correction of the manuscript
Elsayed M Abdelwhab:	Design and supervision of the study; Provide funding; Planning and implementation of the animal trial; Assessment of clinical status in the animal trial and collection of samples; Sequence analysis; Interpretation of data; Main participation in the preparation and correction of the manuscript; Corresponding author

Paper III

Impact of Mutations in the Hemagglutinin of H10N7 Viruses Isolated from Seals on Virus Replication in Avian and Human Cells

Anne Dittrich, David Scheibner, Ahmed H. Salaheldin, Jutta Veits, Marcel Gischke, Thomas C. Mettenleiter and Elsayed M. Abdelwhab

Viruses 2018, 10, 2; DOI: 10.3390/v10020083

Anne Dittrich:	Generation, cloning, mutagenesis and sequencing of expression plasmids coding for gene segments of H10N4turkey and HA of H10N7seal; Rescue, propagation and titration of recombinant viruses; Functional characterization of viruses including replication in CEK and A549 cells; Participation in receptor binding and HA-cleavage studies; Preparation of figures; Participation in the correction of the manuscript
David Scheibner:	HA-glycosylation studies with PNGase F; Participation in HA-cleavage studies; Participation in the correction of the manuscript
Ahmed H. Salaheldin:	Establishment of modified turkey erythrocytes; Conducting the receptor binding studies; Participation in the correction of the manuscript
Jutta Veits:	Participation in the preparation and correction of the manuscript
Marcel Gischke:	Cell-to-cell spread studies; Establishment of solid phase binding assay; Conducting the receptor binding studies; Participation in the correction of the manuscript
Thomas C. Mettenleiter:	Design of the study; Provide funding; Participation in the preparation and correction of the manuscript
Elsayed M. Abdelwhab:	Design and supervision of the study; Sequence analysis; Heat stability studies; Provide funding; Interpretation of Data; Main participation in the preparation and correction of the manuscript; Corresponding author

In agreement (Paper I – III)

Place, Date

Prof. Dr. Dr. h.c. Thomas C. Mettenleiter

Marcel Gischke

5. Discussion

Avian influenza viruses have their natural reservoir in wild aquatic birds and occasionally spread to terrestrial poultry and mammalian hosts. While most of the 16 hemagglutinin subtypes remain low pathogenic in poultry, H5 and H7 viruses can evolve to highly pathogenic viruses by acquiring a polybasic cleavage site in the HA as a major virulence factor (Bosch et al., 1981; Richard et al., 2017). Less is known about the potential requirements of non-H5/H7 viruses to exhibit a highly pathogenic phenotype in poultry. Furthermore, some AIVs were reported to cross species barriers to mammals, partially with fatal outcomes. Although several studies showed that alterations in receptor binding preferences and virus stability mediated by mutations in the HA protein are crucial for AIV adaptation to mammals (Byrd-Leotis et al., 2017; Russier et al., 2016), mutations that confer adaptation of AIVs to aquatic mammals are largely unknown. Therefore, it is important to understand the genetic determinants for virulence and adaptation of non-H5/H7 AIVs in birds and mammals.

In this thesis, the virulence of H4N2 with a natural polybasic HACS was studied in chickens after removing glycosylation sites in the HA stem or adding basic aa in the HACS with and without reassortment with HPAI H5 or H7 viruses (Papers I and II). Furthermore, the role of HA1 mutations in the potential adaptation of H10N7 AIV isolated from seals was investigated *in vitro*. (Paper III).

5.1. Adaptation and virulence of H4N2 in chickens

HPAI H5 and H7 viruses exhibit IVPI > 1.2 and/or polybasic HACS. To date, only a few naturally evolved AIVs of non-H5/H7 subtypes caused lethality in domestic poultry. However, these isolates revealed only moderate pathogenicity after intravenous infections and no or low mortality via natural routes under experimental conditions (Table 2). Moreover, none of these isolates specified a pCS motif and high virulence was only induced after acquisition of an engineered HACS and/or virus passages for some strains (Brugh, 1992; Soda et al., 2011). The emergence of HPAIV H4Nx viruses is more likely than other non-H5/H7 viruses. In 1975, an H4N8 virus caused severe clinical symptoms in a commercial layer flock in Alabama and induced high mortality after consecutive passages in chickens (Brugh, 1992; Slemons and Swayne, 1992). In August 2012, an LPAIV of subtype H4N2 with a four-basic aa HACS motif was isolated from a quail farm in California. Therefore, H4 viruses should be monitored carefully to avoid the emergence of HPAIVs. To assess the potential risk for the transition of this unique virus to HP phenotype, we used two molecular approaches: removal of glycosylation sites in the HA stem domain adjacent to the HACS, or increasing the number of basic amino acids and reassortment with HPAIVs H5/H7.

Deglycosylation of the HA stem described in **Gischke et al. (2021)** increased trypsin-independent replication of H4N2 virus in avian cells, increased cell-to-cell spread and caused broader organ tropism in chicken embryos. Therefore, it is likely that the removal of glycosylation sites in the HA

stem domain enables the access of host-derived proteases for HA proteolytic activation, as seen in different H5 subtypes (Horimoto and Kawaoka, 1995; Kawaoka et al., 1984; Kawaoka and Webster, 1989). In contrast to our results, equivalent glycosylations in other HPAI H5 and H7 viruses are necessary for efficient infectivity and virus replication (Scholtissek, 1985; Wagner et al., 2002a; Yin et al., 2017) and may indicate a strain-dependent effect for N-glycans in the stem domain of AIV on virus fitness. Moreover, and despite the improved replication and dissemination of H4N2 *in ovo*, the sequence analysis revealed high conservation of N-terminal pGS in the HACS vicinity for almost all AIV subtypes including H4Nx viruses **(Gischke et al., 2021)**. These findings suggest the importance of the HA stem glycosylations for thermal and acid stability as reported for HPAI H5 and H7 viruses (Ohuchi et al., 1997a; Yin et al., 2017). It is known that glycosylations stabilize HA at elevated temperatures, e.g., in the intestinal tract of birds, during fever or high ambient temperatures and stabilize the metastable state due to interactions with the oligosaccharides after HA cleavage (Scholtissek, 1985). Notably, the membrane fusion alters intracellular host responses and a rapid fusion at low acidic pH may enhance early virus replication before triggering the host immune response. However, less acid stability affects the persistence *ex vivo* and may influence transmissibility (Russier et al., 2016).

The extension of the naturally evolved pCS by substitutions of T327 (328, H3 numbering), investigated in **Gischke et al. (2020)**, also resulted in a broader organ tropism. However, virus infiltration and tissue damage in chickens remained at lower levels compared to the H4N2 carrying seven gene segments from HPAIV H5N1. The increased tropism due to changing threonine at residue 327 to basic amino acids arginine or lysine was HAT and TMPRSS2 independent and was probably due to HA activation by ubiquitous furin or furin-like proteases by matching the consensus motif of HPAIVs (Abdelwhab et al., 2013; Bosch et al., 1981). In previous studies, it could be shown that some H9N2 viruses with mono-, di- or tribasic HACS can be activated by HAT or TMPRSS2. However, the activation by furin was blocked until the insertion of additional basic aa despite the presence of a consensus sequence (e.g., R-X-R-R) (Baron et al., 2013; Bottcher-Friebertshauser et al., 2013). We found that H4N2 with K327 exhibited broader tropism than H4N2 with R327. The sequence analysis of 38 different cleavage sites revealed a preferential cleavage by furin for motifs carrying K at P2 (Thomas, 2002). These findings suggest that K327 is cleaved by additional or more specific furin-like proteases (Lee et al., 2021; Richard et al., 2017).

Remarkably, the authentic HA4 exhibited an IVPI of 2.1 after reassortment with H5N1 segments and is therefore classified as an HPAIV according to the OIE regulations (Alexander, 2015; OIE, 2021a). However, the same virus caused no mortality and slight morbidity in chickens via oculonasal inoculation. The presence of certain furin-like enzymes in the blood, not in the respiratory tract, which activated H4N2 virus with authentic HACS may explain this observation.

Munster et al. (2010) generated a highly pathogenic H6N1 virus after the introduction of an engineered pCS only. In contrast, as represented in this thesis, neither deglycosylations in the HA stem domain nor additional basic aa in the HACS alone induced high pathogenicity of H4N2 in chickens. Interestingly, additional basic aa in the HACS of H4N2 had a detrimental effect on virus fitness apparent from decreased shedding and low chicken-to-chicken transmission. These results indicate that other non-HA genes contribute to virus fitness and virulence.

Indeed, H4N2 virus fitness was restored after reassortment with segments of HPAIV H5N1 and induced high pathogenicity with massive infiltration and damage in organs of chickens, especially after extension of the natural pCS motif. It has been shown that virulence determinants reside not only in the HA but also in other gene segments of this HPAIV H5N1 (Bogs et al., 2010; Stech et al., 2015). Similarly, HPAIV H5N1 gene segments supported the emergence of HP H2, H4, H6, H8, H9 and H14 subtypes carrying an artificial pCS (Gohrbandt et al., 2011a; Veits et al., 2012), which further confirmed the critical role for additional virulence factors in other viral segments. For instance, H5N1 exhibits adaptive markers like NP S377N (Tada et al., 2011), NS1 deletion 80-84 (Long et al., 2008) or PB1 V14A (Suzuki et al., 2014) are associated with an increased pathogenicity and transmission in chickens. It is highly likely that similar mutations improved the fitness of H4N2 and were necessary to infiltrate the endothelium of vital organs and caused systemic spread with multiorgan dysfunction and eventual death of birds, similar to natural HPAIVs. Conversely, it also suggests that H4N2 is less adapted to chickens than HPAIV H5N1. Accordingly, responsible gene segments and virulence determinants of H5N1, which are required for increased virulence and adaptation of H4N2 in chickens, remain to be identified. In contrast, reassortment with the HPAI H7N7 virus caused no shift in virulence for H4. Scheibner et al. (2019) showed that the polybasic HACS, independent of other gene segments, is the main virulence determinant of this HPAIV H7N7 in chickens. Therefore, non-HA gene segments from HPAIV H7N7 did not increase the virulence of H4N2. Another possible explanation is that H4 and N7 are less compatible and exchanging gene segments e.g. disturbs the essential HA:NA balance for efficient virus replication (de Vries et al., 2020; Wagner et al., 2002b).

The conclusion from Paper I and II is that the removal of N-terminal glycosylations in the vicinity of the HACS or additional basic aa in the pCS of H4N2 alone did not induce high pathogenicity in chickens, neither after intravenous infection nor via the natural route. Although, deglycosylations in **Gischke et al. (2021)** increased the trypsin-independent replication and systemic spread in chicken embryos, they reduced virus stability. Moreover, the extended pCS motif in **Gischke et al. (2020)** compromised the virus excretion and bird-to-bird transmission, which were restored after reassortment with HPAIV H5N1, but not H7N7, indicating a critical role for certain non-HA gene segments in the transition of LP to HP. Given the wide spread of H4 viruses in wild birds and LBMs, and their high reassortment capacity (Teng et al., 2012; Verhagen et al., 2021; Wisedchanwet et al., 2011; Xu et al., 2014), similar H4/H5 genetic constellations might occur in the field.

5.2. Adaptation of H10N7 to seal

Although wild birds are the natural reservoir of AIVs, spill-overs from avian hosts to mammals, including humans, have been reported and occasionally caused fatal infections (Klingeborn et al., 1985; Mostafa et al., 2018; Roberts et al., 2009). Severe AIV outbreaks in seals were repeatedly associated with non-H5/H7 subtypes and caused substantial ecologic losses (Anthony et al., 2012; Callan et al., 1995; Hinshaw et al., 1984).

In the spring and summer of 2014, an H10N7 virus caused mass deaths in harbor and grey seals in northern Europe. In **Dittrich et al. (2018),** we identified prevalent HA1 mutations of H10N7seal and investigated their impact in the fitness of an avian H10N4 virus. The low prevalence of identified H10seal mutations, mainly located in the globular head domain which is known to alter the host range by substitutions of a few residues (Byrd-Leotis, Cummings, and Steinhauer 2017) (Table S1), implies specific adaptive mutations of H10N7 as a result of the interspecies transmission to seal. This assumption is supported by the facts that selective pressure acts first on the surface glycoproteins of IAVs and that previous studies revealed a close relation of H10N7seal to avian H10 viruses (Bodewes et al., 2016; Krog et al., 2015; van den Brand et al., 2016). Therefore, a direct transmission from birds to seals is likely.

A shift in receptor affinity depends on the SA binding orientations onto host cell receptors and depicts a key role for adaptation and interspecies transmission from avian to mammalian hosts (Byrd-Leotis et al., 2017). Interestingly, the receptor preference analysis of H10N7seal unveiled a dual binding affinity, however, with a higher preference for avian-like receptors. Similar results were determined for an avian H3N8 virus that infected harbor seals in 2011 (Hussein et al., 2016; Yang et al., 2015b). Notably, the majority of mammalian and bird H10Nx isolates revealed specificities for avian α2,3 receptors. However, similar to H10N7seal, several strains exhibited a dual receptor binding, some even with a preference for α2,6-SA (Sutton et al., 2017; Vachieri et al., 2014; Wu et al., 2016; Yang et al., 2015a). Remarkably, the glutamine to leucine substitution at position 220 (226, H3 numbering) resulted in the highest increase for α2,6-SA binding, while completely abolishing α2,3-SA affinity and significantly increasing replication in human lung cells. This observation is in accordance with findings from a recent study by Herfst et al. (2020), where L220 enabled H10N7seal a limited ferret-to-ferret transmission. Moreover, several studies identified analogous substitutions in various subtypes, including non-H5/H7 AIVs, as a critical mutation for the shift in the receptor binding preference and for interspecies transmissions to mammalian hosts (Table S1). Q226L often appears in combination with alterations of residue 228 (H3 numbering). Therefore, it is likely that an additional substitution at this position further enhances the α2,6-SA affinity of H10N7seal and improves adaptation to mammalian hosts (Chen et al., 2014; To et al., 2014). At this point, it is worth mentioning that an H10N3 virus has recently spread to humans and its S228 residue indicates a preference for human-like receptors (Wang et al., 2021). Moreover, two mutations of H10seal disrupted the GS motif ^{236}NIT^{238}. The loss of glycosylation in the head

domain, e.g., at N133 or N158 (H3 numbering), increased the binding to α2,6-SA for some H5, H7 and H9 viruses (Chang et al., 2020; Lee et al., 2018; Wang et al., 2010). Conversely, substituting the glycosylated N236 (242, H3 numbering) residue revealed no impact on the α2,6-SA affinity and suggests another role of I238 (244, H3 numbering) for the increased binding. Equivalents to the avian T238 and T165 (also altered in H10seal) were recently predicted to facilitate the intrasubunit contact for H2 and H6 HAs and may increase the availability for glycan-modifying enzymes to N-glycans in the head domain during maturation. The improved access may lead to more complex compositions than high mannose glycan structures, which might affect the receptor binding preference. On the contrary, high mannose structures foster the virus removal by the pulmonary surfactant protein D from the lungs similar to human strains (Parsons et al., 2020). Furthermore, additional residues of H10seal, e.g., S113N (122, H3 numbering), also increased the affinity to α2,6-SA. A similar mutation was found for an H5N1 vaccine virus and contributed to its dual receptor specificity (Wang et al., 2010). In contrast to H10N7seal, the H10N8 virus that caused a fatal human case in China maintained the avian-like receptor binding preference. However, it expressed other mammalian adaptation markers in HA, M1, NS1 and PB2 (Chen et al., 2014; To et al., 2014). These findings indicate that despite the important role of an altered receptor binding affinity, other biological features are necessary for the efficient transmission of AIVs to mammals.

One of the essential properties required for efficient replication of AIVs in mammals is thermostability and HA cleavability (Herfst et al., 2012; Sutton et al., 2014). Besides the affinity to α2,6-SA, especially two H10seal residues induced a remarkably increased stability at elevated temperatures, which may contribute to the spread between seals. Previous reports revealed a contribution to virulence by non-basic aa adjacent to the cleavage site (Blaurock et al., 2020; Gohrbandt et al., 2011b). However, V321 in the HACS of H10seal had no role for proteolytic activation in MDCKII cells. Notably, the hypothetic cleavability by proteases apart of MDCKII cannot be excluded. For instance, HAT and TMPRSS2 were found to cleave the V-S-S-R motif of H9, which is remotely similar to the HACS of H10N7 (Baron et al., 2013). Moreover, a recent study with TMPRSS2 deficient mice determined a critical role for this protease in the proteolytic activation of H10 viruses (Lambertz et al., 2019).

The generally lower replication in human lung cells shows a poor adaptation of H10N7seal to mammalian host cells. Notably, I238 played a critical role in the reduced replication at temperatures resembling conditions in the upper respiratory tract of mammals (Suttie et al., 2019). According to Herfst et al. (2020), this substitution occurred, together with an E to D exchange in HA2 at position 74 (HA0: 398; 403, H3 numbering) in late infected seals, suggesting an ongoing adaptation of H10N7 during the outbreak. This may explain the relatively low prevalence for T238I between the considered isolates in **Dittrich et al. (2018)**. Interestingly, both substitutions enhanced the acid stability of HA similar to pandemic strains and facilitated efficient transmission in ferrets. It is known that acid-induced changes are related to the thermal stability of HA (Remeta et al., 2002).

Accordingly, the increased stability facilitated by I238 (and D398) may stabilize the metastable state upon cleavage and acidification of HA at lower temperatures, and could explain the significantly reduced replication at 33°C. These findings suggest adaptations of H10N7seal to increase HA stability and subsequently facilitate efficient transmission between mammalian hosts (Russier et al., 2016) due to a better persistence *ex vivo*, but at high fitness costs. Additional mutations in the HA or other gene segments could restore the low viral fitness in mammals (Suttie et al., 2019). The genome sequencing of mammal-adapted H10 viruses revealed several adaptation markers like PB2 E627K, PA T97I and HA G409E (Wu et al., 2016; Zhang et al., 2016). For example, H10N7seal maintained E627 in PB2. A substitution would possibly lead to an increased replication, especially at lower temperatures in the upper respiratory tract of mammals and raise the zoonotic potential of this non H5/H7 AIV

In conclusion, H10N7seal developed a dual receptor binding specificity mainly forced by the mammalian adaptation marker L220 (226, H3 numbering). However, it remained more adapted to avian host cells. Nevertheless, the occurrence of this substitution suggests a stepwise adaptation from birds to seals. Although controversially discussed, seals may act as mixing vessels (Anthony et al., 2012; Ito et al., 1999) and consequently, could give rise for viruses with zoonotic or even pandemic potential, like the pandemic H1N1 virus in 2009 (Russier et al., 2016). Additionally, further mutations were identified to modulate receptor binding specificity and virus stability, and possibly contributing to adaptation in the mammalian hosts. Therefore, H10N7seal is a representative example how AIVs can adapt to and evolve in aquatic mammals.

6. Summary

Avian influenza viruses (AIVs) have their natural reservoir in wild aquatic birds but occasionally spread to terrestrial poultry. While AIVs of subtypes H5 and H7 are well known to evolve highly pathogenic avian influenza viruses (HPAIVs) during circulation in domestic birds, non-H5/H7 subtypes exhibit only a low to moderate pathogenicity. Furthermore, spillover events to a broad range of mammalian hosts, including humans, with self-limiting to severe illness or even fatal outcomes, were reported for non-H5/H7 AIVs and pose a pandemic risk. The evolution of high virulent phenotypes in poultry and the adaptation of AIVs to mammalian hosts are predominantly linked to genetic determinants in the hemagglutinin (HA). The acquisition of a polybasic cleavage site (pCS) is a prerequisite for the evolution of HPAIVs in poultry, while changes in the receptor binding preference and virus stability are essential for adaptation of AIVs to mammals.

In August 2012, an H4N2 virus with the pCS motif ^{322}PE**KRR**T**R**/G^{329} but preserved trypsin dependend replication and low pathogenicity in chickens was isolated on a quail farm in California. In the first two publications, we followed different approaches to investigate virulence factors and the potential risk for the transition of H4N2 to high virulence in chickens. The loss of N-terminal glycosylations in the vicinity of the pCS resulted in decreased binding to avian-like receptors and dramatically decreased virus stability. On the other hand, one deglycosylation increased virus replication and tissue tropism in chicken embryos but did not alter virulence or excretion in chickens. Furthermore, additional basic amino acids in the natural pCS motif improved the trypsin-independent cleavage of HA and caused slightly increased tissue tropism in chickens. However, the engineered motifs alone did not affect virulence in chickens. Intriguingly, they even had a detrimental effect on virus fitness, which was restored after reassortment with segments of HPAIV H5N1. Together, the results show the importance of HA glycosylations on the stability of H4N2 and reveal the important role of non-HA segments in the transition of this virus to high virulence in poultry.

The transmission of another non-H5/H7 AIV of subtype H10N7 from birds to seals resulted in mass deaths in harbor seals in 2014 in northern Europe. The third publication describes nine mutations in the HA1 subunit of seal isolates compared to avian H10Nx viruses. We found that some of these mutations conferred a dual specificity for avian and mammalian receptors and altered thermostability. Nevertheless, the H10N7seal remained more adapted to avian host cells, despite of the alteration in the receptor binding specificity.

Altogether, this thesis demonstrates that naturally evolved AIVs beside H5 and H7 subtypes support a highly pathogenic phenotype in the appropriate viral background and alter virulence and host receptor specificity by few amino acid substitutions in the HA. These findings improve our knowledge of the potential of non-H5/H7 AIVs to shift to high virulence in birds and the adaptation in mammals.

7. Zusammenfassung

Aviäre Influenzaviren (AIVs) haben ihr natürliches Reservoir in wilden Wasservögeln, werden jedoch auch auf Landgeflügel übertragen. Im Gegensatz zu AIVs der Subtypen H5 und H7, welche hochpathogene aviäre Influenzaviren (HPAIVs) während der Zirkulation in domestizierten Vögeln entwickeln können, verursachen nicht-H5/H7 Subtypen lediglich eine niedrige bis moderate Pathogenität. Infektionen von Säugetieren, darunter Menschen, mit nicht-H5/H7 AIVs führten zudem zu selbstlimitierenden bis schweren Krankheitsverläufen teils mit tödlichen Folgen und stellen somit ein pandemisches Risiko dar. Die Entwicklung hochvirulenter Phänotypen in Geflügel und die Anpassung von AIVs an Säugetierwirte sind vorrangig auf genetische Faktoren im Hämagglutinin (HA) zurückzuführen. Der Erwerb einer polybasischen Spaltstelle (pCS) ist eine Voraussetzung für die Bildung von HPAIVs in Geflügel, wohingegen Veränderungen der Rezeptorbindungspräferenz und der Virusstabilität für die Anpassung an Säuger maßgeblich sind.

Im August 2012 wurde ein H4N2 Virus mit dem pCS Motiv ^{322}PE**KRR**T**R**/G^{329} auf einer Wachtelfarm in Kalifornien isoliert, welches jedoch weiterhin Trypsin-abhängig replizierte und eine niedrige Pathogenität in Hühnern aufwies. In den ersten beiden Publikationen verfolgten wir verschiedene Ansätze zur Untersuchung von Virulenzfaktoren und dem Potenzial von H4N2, eine hohe Virulenz in Hühnern zu induzieren. Der Verlust N-terminaler Glykosylierungen in Umgebung der pCS führte zu reduzierten Bindungspräferenzen für vogelartige Rezeptoren und geringen Virusstabilitäten. Eine Deglykosylierung führte zu einer verbesserten Virusreplikationen und verbreiterten Gewebetropismen in Hühnerembryonen, hatte jedoch keinen Einfluss auf die Virulenz oder Ausscheidung in Hühnern. Zusätzliche basische Aminosäuren im natürlichen pCS Motiv führten zu Trypsin-unabhängigen HA-Spaltungen und gesteigerten Gewebetropismen, hatten allein aber keinen Einfluss auf die Virulenz und sogar einen nachteiligen Effekt auf die Virusfitness, welche allerdings durch Reassortierung mit HPAIV H5N1 Segmenten wiederhergestellt wurden. Die Ergebnisse zeigen sowohl die Bedeutung von HA-Glykosylierungen für die Stabilität von H4N2, als auch die Relevanz für Nicht-HA Segmente bei der Ausbildung hoher Virulenzen auf. Die Transmission von Vögeln auf Seehunde eines anderen nicht-H5/H7 AIV vom Subtyp H10N7 führte 2014 zu einem Massensterben von Seehunden in Nordeuropa. Die dritte Publikation beschreibt neun Mutationen der HA1-Untereinheit in Seehundisolaten im Vergleich zu aviären H10Nx Viren. Wir fanden heraus, dass einige dieser Mutationen eine duale Spezifität für aviäre und Säugetierrezeptoren verleihen und die Thermostabilität verändern. Trotz der veränderten Rezeptorspezifität ist H10N7seal jedoch weiterhin stark an aviäre Wirtszellen adaptiert.

Zusammengefasst demonstriert die vorliegende Arbeit, dass natürliche AIVs, neben H5 und H7 Subtypen, einen hochpathogenen Phänotyp im geeigneten viralen Hintergrund unterstützen und die Virulenz sowie die Wirtsrezeptorspezifität durch Substitution weniger Aminosäuren im HA beeinflusst werden. Diese Forschungsergebnisse verbessern unsere Kenntnisse zum Potenzial von Nicht-H5/H7 AIVs, hohe Virulenzen in Vögeln zu entwickeln und an Säugetiere zu adaptieren.

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9. Appendix

9.1. Supplementary

Table S1: Adaptive hemagglutinin mutations found to modulate replication, transmission and virulence in mammals

Mutation **H3 numbering**	**Phenotype**	**Subtype**	**Host** **Isolated /tested**	**References**
Y17H	Increase pH of fusion, decreased HA stability, Decrease virulence in mice and ferrets	H1N1	Human isolate, Mice, Ferrets	(Russier et al., 2016)
Y23H	Increase pH of fusion, decreased HA stability, Decrease virulence in mice	H5N1	Mice	(Zaraket et al., 2013b)
H24Q	Decrease pH of fusion, increased HA stability	H5N1	Mice	(Zaraket et al., 2013b)
D101N	Increase affinity to α2,6-SA, increase fusion activity in 293T cells	H5N1	Human isolate	(Su et al., 2008)
S125N	Increase affinity to α2,6-SA (dual receptor specificity)	H5N1	Ferrets	(Wang et al., 2010)
N133T	Increase affinity to α2,3 and α2,6-SA due to loss of glycosylation Decrease pathogenicity in mice	H9N2	*In ovo* adaptation, Mice	(Lee et al., 2018)
A135T	Decrease affinity to α2,6-SA due to gain of glycosylation	H7N9	Immune escape mutant in ferrets	(Chang et al., 2020)
S137A	Increase affinity to α2,6-SA	H5N1	Human isolate	(Yang et al., 2007)
A137V	Increase affinity to α2,6-SA, increase virulence in mice	H5N8	Adaptation in mice after passaging	(Wu et al., 2017)
A138S	Increase affinity to α2,6-SA	H5N1 H7N9	Swine isolate Human isolate	(Nidom et al., 2010) (Kageyama et al., 2013)
A138V	Increase affinity to α2,6-SA	H5N1	Human isolate	(Kongchanagul et al., 2008; Naughtin et al., 2011)

G143R	Increase affinity to α2,6-SA	H5N1	Human isolate	(Yamada et al., 2006)
I155T	Increase affinity to α2,6-SA	H5N1 H9N2	Human isolate, Mice	(Watanabe et al., 2011) (Li et al., 2014)
G/S158N	Decrease affinity to α2,6-SA when glycosylated enhance viral productivity, exacerbate the host response in mice	H5N1	Ferrets, Mice	(Ilyushina et al., 2008; Wang et al., 2010; Zhao et al., 2017)
N158D	Decrease virulence in mice	H9N2	Immune escape mutant in mice	(Jin et al., 2019)
S159N	Increase affinity to α2,6-SA	H5N1	Ferrets	(Wang et al., 2010)
T160A	Increase affinity to α2,6-SA, Increase transmission in guinea pigs, loss of glycosylation at N158	H5N1	Human isolate, Mice, Ferrets, Guinea pigs	(Chen et al., 2007; Gao et al., 2009; Wang et al., 2010)
A160T	Decrease affinity to α2,6-SA due to gain of glycosylation Decrease thermal HA-stability	H7N9	Immune escape mutant in ferrets	(Chang et al., 2020)
H183N	Decrease affinity to α2,3-SA and α2,6-SA	H9N2	Poultry isolate	(Li et al., 2014; Matrosovich et al., 2001)
G186V	Increase affinity to α2,3-SA	H7N9	Human isolate	(Dortmans et al., 2013)
N186K/D	Increase affinity to α2,6-SA	H5N1	Human isolate, Ferrets, Guinea pigs	(Chutinimitkul et al., 2010b; Gao et al., 2009; Kongchanagul et al., 2008; Yamada et al., 2006)
V186N	Increase affinity to α2,6-SA, Decrease affinity to α2,3-SA	H13N6	-	(Lu et al., 2013)
P186L	Decrease affinity to α2,3-SA	H6N1	Human isolate	(Wang et al., 2015)
D187G	slightly increase affinity to α2,6-SA	H5N1	*in vitro* isolation	(Chen et al., 2012)
E190D	Increase affinity to α2,6-SA	H1N1	Human isolate	(Glaser et al., 2005)
E190G	Slightly increase affinity to α2,6-SA, Maintain affinity to α2,3-SA; Decrease virulence in mice	H5N1	*in vitro* isolation, Mice	(Chen et al., 2012) (Han et al., 2015)

E190V	Increase affinity to α2,6-SA Decrease affinity to α2,3 and α2,6-SA	H6N1 H6N2	Human isolate Guinea pig	(Wang et al., 2015) (Qu et al., 2017)
T190V	Enhanced binding affinity to and replication in mammalian cells (no change in specificity to α2,6-SA)	H9N2	Mice	(Teng et al., 2016)
A/I190E/D	Increase affinity to α2,6-SA	H9N2	Human isolate	(Peacock et al., 2017)
T192I	Increase affinity to α2,6-SA	H5N1	Human isolate	(Yang et al., 2007)
K193R/T	Increase affinity to α2,6-SA (dual receptor specificity) Transmission among ferrets	H5N1	Ferrets	(Wang et al., 2010) (Peng et al., 2018)
Q196R/H	Increase affinity to α2,6-SA Decrease affinity to α2,3-SA	H5N1	*in vitro* isolation, Human isolate	(Chen et al., 2012; Gao et al., 2009; Watanabe et al., 2011; Yamada et al., 2006)
N197K	Increase affinity to α2,6-SA	H5N1	Human isolate	(Yamada et al., 2006)
V214I	Increase affinity to α2,6-SA	H5N1	Human isolate	(Watanabe et al., 2011)
D222G	Increase affinity to α2,6-SA (dual receptor specificity)	H1N1	Human isolate	(Chutinimitkul et al., 2010a)
G225D	Increase affinity to α2,6-SA Complete loss of affinity to α2,3-SA	H1N1 H6N1	Human isolate	(Glaser et al., 2005) (de Vries et al., 2017b)
Q226L	Increase affinity to α2,6-SA Airborne transmission among guinea pigs Strain dependent increased or decreased affinity to α2,6-SA Increase affinity to α2,3-SA Decrease affinity to α2,6-SA	H4N6 H6N2 H5N1 H7N9	Swine isolate Guinea pig Human isolate Human isolate	(Song et al., 2017) (Qu et al., 2017) (Chutinimitkul et al., 2010b; Stevens et al., 2006) (Li et al., 2014; Ramos et al., 2013; Sang et al., 2015; Wan and Perez,

				2007; Wan et al., 2008; Xu et al.,
	Increase affinity to α2,6-SA	H9N2	Ferrets	2013)
	Enhance replication in mammalian cells and ferrets		Adaptation in mice after	(Herfst et al., 2020)
	Contact transmission among ferrets		passaging Guinea pig	(Tzarum et al., 2017; Zhang et al.,
	Increase affinity to α2,6-SA	H10N7	Seal isolate	2015a)
	Decrease affinity to α2,3-SA, no affinity to α2,6-SA	H10N8	Human isolate	
L226Q	Increase affinity to α2,3-SA	H7N9	Immune escape mutant	(Chang et al., 2020)
	Enhanced acid and thermal HA-stability		in ferrets	
L226I	Decrease affinity to α2,3-SA	H7N9	Human isolate	(Xu et al., 2013)
S227N/Q	Increase affinity to α2,6-SA	H5N1	*in vitro* isolation, Human	(Chen et al., 2012; Chutinimitkul et
			isolate	al., 2010b; Gambaryan et al., 2006;
				Kongchanagul et al., 2008)
		H9N2	Human isolate	(Sun et al., 2020)
G228A/S	Increase affinity to α2,6-SA (dual receptor specificity)	H4N6	Swine isolate	(Song et al., 2017)
G228S	Increase replication in mammalian cells and mice	H1N2	Adaptation in mice after	(Yu et al., 2019)
			passaging	
	Increase affinity to α2,6-SA	H5N1	Human isolate	(Chutinimitkul et al., 2010b;
	Decrease affinity to α2,3-SA			Stevens et al., 2006; Wang et al.,
				2010)
		H6N2	Guinea pig	(Qu et al., 2017)
	Decrease affinity to α2,3-SA and 2.6 SA	H6N1		(Wang et al., 2015)
		H7N9	Human isolate	(de Vries et al., 2017a)
		H10N3	Human isolate	(Wang et al., 2021)
	Decrease affinity to α2,3-SA, no affinity to α2,6-SA	H10N8	Human isolate	(Zhang et al., 2015a)
S239P	Slightly increase affinity to α2,6-SA	H5N1	Human isolate	(Watanabe et al., 2011)

E255K	Increase affinity to α2,6-SA	H5N1	*in vitro* isolation	(Chen et al., 2012)
K/R304R	Airborne transmission among ferrets	H7N1	Adaptation in ferrets after passaging	(Sutton et al., 2014)
326 – 329	pCS Increase virus replication, pathogenicity and transmission Increase virulence in mice, impact on HA stability	H5Nx H7Nx H9N2	Human isolate, Ferrets, Mice Human isolate, Ferrets, Mice, Mice	(Schrauwen et al., 2012; Subbarao et al., 1998; Suguitan et al., 2012; Zhang et al., 2012) (Chan et al., 2020; Sun et al., 2016; Sun et al., 2019; Zhu et al., 2017) (Zhang et al., 2021)
K387I	Decrease pH of fusion, increased HA stability Increase replication efficiency and virulence in mice and ferrets	H5N1	Mice, Ferrets	(Krenn et al., 2011; Zaraket et al., 2013a; Zaraket et al., 2013b)
K393E	Increased pH of fusion, decreased HA stability Decreased virulence in mice	H7N9	Human isolate, Mice	(Sun et al., 2019)
G409E	Increase virulence in mice	H10N7	Adaptation in mice after passaging	(Wu et al., 2016)
F430L	Increase virulence in mice	H5N5	Adaptation in mice after passaging	(Yu et al., 2018)
Combination of mutations that are linked to mammalian adaptation				
H17Y, R435K	Decrease pH of fusion, increased HA stability Increase replication efficiency and virulence in ferrets Cause airborne transmission in ferrets	H1N1	Ferret adapted revertant	(Russier et al., 2016)
P78L, H354Q	Decrease pH of fusion, increased HA stability Increase virulence in mice	H1N1	Mice	(Koerner et al., 2012)
E83K, S128P, N197K, R496K	Increase affinity to α2,6-SA	H5N1	Human isolate	(Yamada et al., 2006)

H110Y, T160A, Q226L, G228S	Increase affinity to α2,3-SA and α2,6-SA Airborne transmissible among ferrets	H5N1	Adaptation in ferrets after passaging	(Herfst et al., 2012; Linster et al., 2014; Stevens et al., 2008; Wang et al., 2010)
I111T, A146S, pCS	Increased virulence in mice	H7N7	Adaptation in ferrets after passaging, Mice	(Dreier et al., 2019)
S114R, T115I	Increased virulence in mice (and chicken), Increased pH of fusion	H5N1	Mice	(Wessels et al., 2018)
L129V (H5 numbering)* A138V	Increase affinity to α2,6-SA	H5N1	Human isolate	(Auewarakul et al., 2007)
L129del (H5 numbering)*, I155T	Increase affinity to α2,6-SA	H5N1	Human isolate, Mice	(Watanabe et al., 2011)
A135T, A160T	Loss of α2,6 binding ability	H7N9	Immune escape mutant in ferrets	(Chang et al., 2020)
S137A, T192I	Increase affinity to α2,6-SA	H5N1	Human isolate	(Yang et al., 2007)
G143R, N186K Q196R, Q226L, S227N, G228S	Decrease affinity to α2,3-SA Increase affinity to α2,6-SA	H5N1	Determination by sequence alignment	(Chen et al., 2012; Chutinimitkul et al., 2010b; Yamada et al., 2006)
H156N, S263R	Increase virulence in mice	H6N6	Adaptation in mice	(Tan et al., 2014)
N158D, N224K, Q226L, T318I	Droplet transmissible among ferrets	H5N1 (in background of pH1N1)	Adaptation in Ferrets	(Imai et al., 2012)
N158S, Q226L, N248D	Increase affinity to α2,6-SA	H5N1	-	(Ilyushina et al., 2008)

S159N, T160A, S227N	Increase affinity to α2,6-SA Decrease virulence in mice	H5N1	Human isolate, Ferrets, Mice	(Wang et al., 2010; Yen et al., 2009)
T160A, K193T, N224K, Q226L	Increase affinity to α2,6-SA	H5N1	Ferrets	(Peng et al., 2018)
P186L, E190V, G228S	Decrease affinity to α2,3-SA , Increase affinity to α2,6-SA	H6N1	Human isolate	(Wang et al., 2015)
V186N/K; K/G, K193T, N224K, N/G228K/S	Increase affinity to α2,6-SA (dual receptor specificity)	H7N9	Determination by sequence alignment	(de Vries et al., 2017a)
E187G, E190D, K193R/S, Q226L, G228S	Increase affinity to α2,6-SA	H5N1	Determination by sequence alignment	(Maines et al., 2011; Stevens et al., 2008)
E187G, E190G, Q226E/L, S227N, G228S	Increase affinity to α2,6-SA	H5N1	*in vitro* isolation	(Chen et al., 2012)
T189A, G192R	Enhanced replication in ferrets, droplet transmisable among ferrets	H9N2 (background of human H3N2)	Adaptation in Ferret	(Sorrell et al., 2009)
A190V, T212I	Increase affinity to α2,6-SA Increase virulence in mice	H9N2	*In vitro* isolation, Mice	(Yang et al., 2017)
V216G, E439D	Increased replication in mice	H9N2	*In ovo* adaptation, Mice	(Lee et al., 2018)
K222Q, S227R	Increase a2.3 and a2.6	H5N1, H5Nx	Determination by sequence alignment	(Guo et al., 2017)

Q226L, G228S	Increase affinity to α2,6-SA Increase affinity to α2,6-SA Decrease virulence in guinea pigs	H4N6 H5N1	Swine isolate Guinea Pig	(Song et al., 2017) (Ayora-Talavera et al., 2009; Chen et al., 2012; Chutinimitkul et al., 2010b; Gao et al., 2009; Harvey et al., 2004; Ilyushina et al., 2008; Maines et al., 2011; Stevens et al., 2008; Stevens et al., 2006; Wang et al., 2010)
	Increase affinity to α2,6-SA Loss of affinity to α2,3-SA, no gain of affinity to α2,6-SA	H7N7 H7N9 H10N8H10N8	Human isolate Human isolate Human isolate	(Srinivasan et al., 2013) (Ramos et al., 2013) (Tzarum et al., 2017) (Zhang et al., 2015a)
Q227P, D375E	Increase affinity to α2,3-SA α2,6-SA Transmissible among guinea pigs (D375E increase thermostability)	H9N2	Adaptation in guinea pigs	(Sang et al., 2015)
T244I, E403D	Decreased pH of fusion, increased HA stability	H10N7	Seal isolate	(Herfst et al., 2020)
L331I, G453R	Increase virulence in mice	H4N6	Adaptation in mice after passaging	(Xu et al., 2020)
G396S, S442F	Enhance the pH-dependent, HA membrane fusion	H1N1	Adaptation in swine after passaging	(Bourret et al., 2017)

*Residue deleted in A/H3

9.2. Eigenständigkeitserklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbstständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Marcel Gischke

9.3. Curriculum vitae

9.4. Publications

9.4.1. Publications with contribution to the thesis

Dittrich, A., Scheibner, D., Salaheldin, A.H., Veits, J., Gischke, M., Mettenleiter, T.C., Abdelwhab, E.M. 2018. Impact of Mutations in the Hemagglutinin of H10N7 Viruses Isolated from Seals on Virus Replication in Avian and Human Cells. *Viruses*, 10(2). doi: 10.3390/v10020083

Gischke, M., Ulrich, R., Fatola, O.I., Scheibner, D., Salaheldin, A.H., Crossley, B., Böttcher-Friebertshäuser, E., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M. 2020. Insertion of Basic Amino Acids in the Hemagglutinin Cleavage Site of H4N2 Avian Influenza Virus (AIV)—Reduced Virus Fitness in Chickens is Restored by Reassortment with Highly Pathogenic H5N1 AIV. *Int. J. Mol. Sci.*, 21(7). doi: 10.3390/ijms21072353

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9.4.2. Publications without contribution to the thesis

Hofmann, R., Diniz, P., Pahl, R., Gischke, M., Schwarzer, Y. (2016). Beachtung möglicher Einflussparameter. Betrachtung der klassischen Forciermethode zur Vorhersage der Trübungsstabilität. *BRAUINDUSTRIE* 101(3), 14 – 18

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Scheibner, D., Ulrich, R., Fatola, O.I., Graaf, A., Gischke, M., Salaheldin, A.H., Harder, T.C., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M. (2019). Variable Impact of the Hemagglutinin Polybasic Cleavage Site on Virulence and Pathogenesis of Avian Infuenza H7N7 Virus in Chickens, Turkeys and Ducks. *Scientific Reports*, 47(17). doi: 10.1093/nar/gkz627

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Landmann, M., Scheibner, D., Graaf, A., Gischke, M., Koethe, S., Fatola, O.I., Raddatz, B., Mettenleiter, T.C., Beer, M., Grund, C., Harder, T., Abdelwhab, E.M., Ulrich, R. (2021) A Semiquantitative Scoring System for Histopathological and Immunohistochemical Assessment of Lesions and Tissue Tropism in Avian Influenza. *Viruses*, *13*(5). DOI: 10.3390/v13050868

Salaheldin, A.H., Scheibner, D., Bagato, O., Zaeck, L.M., Mostafa, A., Blohm, U., Müller, C., Eweas, A.F., Gischke, M., Hoffmann, D., Lerolle, S., Li, X., Abd-El-Hamid, H.S., Veits, J., Breithaupt, A., Finke, S.; Pleschka, S.; Mettenleiter, T.C., Abdelwhab, E.M. The neuraminidase of human-origin avian influenza H5N1 viruses plays diverse roles in virus adaptation to mammals *submitted* July 2021, PLOS Pathogens

9.5. Scientific presentations

Mikrobiologisches Kolloquium (Winterkolloquium) der Landesexzellenzinitiative M-V, 28. Jan. 2021, Greifswald, Germany, Oral presentation (online). *Biofilm formation on novel implant materials and microbiome profiling.* Gischke, M., Riedel, K.

13th Annual Meeting EPIZONE, 26 – 28 Aug. 2019, Berlin, Germany, Oral presentation by E.M. Abdelwhab, *Evolution of highly pathogenic non-H5/H7 virus from a natural H4N2 avian influenza virus with a polybasic cleavage motif.* Gischke, M., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

13th Annual Meeting EPIZONE, 26 – 28 Aug. 2019, Berlin, Germany, Poster presentation by C. Blaurock, *Reassortment with H9N2 or NS1-mutations increased virulence of avian-influenza-H5N8 2.3.4.4 in mice but compromised virus replication, virulence and/or transmission in chickens.* Blaurock, C., Scheibner, D., Gischke, M., Mettenleiter, T.C., Abdelwhab, E.M.

13th Annual Meeting EPIZONE, 26 – 28 Aug. 2019, Berlin, Germany, Poster presentation by E.M. Abdelwhab. *Mutations in conserved neuraminidase residues of avian influenza H5N1 virus naturally isolated from humans modulated sialidase activity and virulence in mice but not in chickens* Salaheldin, A.H., Blohm, U., Gischke, M., Scheibner, D., Hoffmann, D., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

13th Annual Meeting EPIZONE, 26 – 28 Aug. 2019, Berlin, Germany, Poster presentation by D. Scheibner, *Virulence Determinants of a German Avian Influenza Virus Isolate Subtype H7N7 in different poultry species.* Scheibner, D., Ulrich, R.G., Fatola, O.I., Gischke, M., Veits, J., Blohm, U., Mettenleiter, T.C., Abdelwhab, E.M.

7th European Congress of Virology, 28. Apr. – 01. Mai 2019, Rotterdam, Netherlands, Poster presentation by E.M. Abdelwhab, *Three different genetic constellations for the emergence of highly pathogenic (HP) non-H5/H7 virus from a natural HP H4N2 avian influenza virus.* Abdelwhab, E.M., Gischke, M., Veits, J., Mettenleiter, T.C.

29th Annual Meeting of the Society for Virology, 20 – 23 Mar. 2019, Düsseldorf, Germany, Poster presentation. *High virulence of a natural H4N2 avian influenza virus with a polybasic cleavage motif after mutation in the hemagglutinin, reassortment with highly pathogenic H5N1 virus or serial passages in eggs.* Gischke, M., Winter, F., Ulrich, R.G., Scheibner, D., Fatola, O.I., Salaheldin, A.H., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

29th Annual Meeting of the Society for Virology, 20 – 23 Mar. 2019, Düsseldorf, Germany, Poster presentation by E.M. Abdelwhab. *Mutations in conserved neuraminidase residues of avian influenza H5N1 virus naturally isolated from humans modulated sialidase activity and virulence in mice but not in chickens* Salaheldin, A.H., Blohm, U., Gischke, M., Scheibner, D., Hoffmann, D., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

29th Annual Meeting of the Society for Virology, 20 – 23 Mar. 2019, Düsseldorf, Germany, Poster presentation by C. Blaurock. *Polymorphism in the hemagglutinin proteolytic cleavage site of H9N2 affected proteolytic activation and cell-to-cell spread in cell culture and virus excretion in infected chickens*. Blaurock, C., Scheibner, D., Gischke, M., Mettenleiter, T.C., Abdelwhab, E.M.

29th Annual Meeting of the Society for Virology, 20 – 23 Mar. 2019, Düsseldorf, Germany, Poster presentation by D. Scheibner. *Virulence determinants of a recent H7N7 avian influenza virus in chickens.* Scheibner, D., Salaheldin, A.H., Gischke, M., Winter, F., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

FLI Junior Scientist Symposium, 24 - 26 Sep. 2018, Greifswald, Germany, Poster presentation. *High virulence of a natural H4N2 avian influenza virus with a polybasic cleavage motif after mutation in the hemagglutinin or reassortment with highly pathogenic H5N1 virus.* Gischke, M., Winter, F., Scheibner, D., Salaheldin, A.H., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

FLI Junior Scientist Symposium, 24 - 26 Sep. 2018, Greifswald, Germany, Poster presentation by D. Scheibner. *Virulence determinants of a recent H7N7 avian influenza virus in chickens.* Scheibner, D., Salaheldin, A.H., Gischke, M., Winter, F., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

FLI Junior Scientist Symposium, 24 - 26 Sep. 2018, Greifswald, Germany, Poster presentation by F. Winter. *Investigation of virulence determinants in highly pathogenic H10 avian influenza viruses using reverse genetics and animal experiments.* Winter, F., Gischke, M., Dittrich, A.; Veits, J.; Mettenleiter, T.C., Abdelwhab, E.M.

10th International Symposium on Avian Influenza, 15 - 18 Apr. 2018, Brighton, UK, Poster presentation. *High virulence of a natural H4N2 avian influenza virus with a polybasic cleavage motif after mutation in the hemagglutinin or reassortment with highly pathogenic H5N1 virus.* Gischke, M., Winter, F., Scheibner, D., Salaheldin, A.H., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

10th International Symposium on Avian Influenza, 15 - 18 Apr. 2018, Brighton, UK, Oral presentation by D.Scheibner. *Pathogenicity and virulence determinants of recent German H7N7 viruses in different poultry species.* Scheibner, D., Salaheldin, A.H., Winter, F., Gischke, M., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

10th International Symposium on Avian Influenza, 15 - 18 Apr. 2018, Brighton, UK, Poster presentation by E.M. Abdelwhab, *Genetic charcterisation, virulence and adaptation markers in historic and recent European H10Nx viruses in-vivo and in-vitro*. Abdelwhab, E.M., Dittrich, A., Winter, F., Blohm, U., Salaheldin, A.H., Gischke, M., Scheibner, D., Veits, J., Mettenleiter, T.C.,

28th Annual Meeting of the Society for Virology, 14 – 17 Mar. 2018, Würzburg, Germany, Poster presentation. *High virulence of a natural H4N2 avian influenza virus with a polybasic cleavage motif after mutation in the hemagglutinin or reassortment with highly pathogenic H5N1 virus.* Gischke, M., Winter, F., Scheibner, D., Salaheldin, A.H., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

28th Annual Meeting of the Society for Virology, 14 – 17 Mar. 2018, Würzburg, Germany, Poster presentation by D. Scheibner. *Virulence determinants of a recent H7N7 avian influenza virus in chickens.* Scheibner, D., Salaheldin, A.H., Gischke, M., Winter, F., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

28th Annual Meeting of the Society for Virology, 14 – 17 Mar. 2018, Würzburg, Germany, Poster presentation by A. Dittrich. *Virulence and adaptation markers in historic and recent European H10Nx viruses in vivo and in vitro.* Dittrich, A. Blohm, U., Winter, F., Salaheldin, A.H., Gischke, M., Scheibner, D., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

1st International Conference on Respiratory Pathogens, 1 – 3 Nov. 2017, Rostock, Germany, Poster presentation. *Determination of potential virulence of an AIV from subtype H4N2 with a polybasic cleavage motif within the hemagglutinin using reverse genetics and animal experiments.* Gischke, M., Winter, F., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

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1st International Conference on Respiratory Pathogens, 1 – 3 Nov. 2017, Rostock, Germany, Poster presentation by A. Salaheldin. *Mutations in conserved NA residues of H5N1 naturally isolated from humans modulated sialidase activity and virulence in mice but not in chickens.* Salaheldin, A.H., Blohm, U., Scheibner, D., Gischke, M., Hoffmann, D., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

1st International Conference on Respiratory Pathogens, 1 – 3 Nov. 2017, Rostock, Germany, Poster presentation by F. Winter. *Investigation of virulence determinants in highly pathogenic H10 avian influenza viruses using reverse genetics and animal experiments.* Winter, F., Gischke, M., Dittrich, A.; Veits, J.; Mettenleiter, T.C., Abdelwhab, E.M.

FLI Junior Scientist Symposium, 20 – 22 Sep. 2017, Braunschweig, Germany, Oral and Poster presentation. *Virulence of avian influenza H4N2 virus in chickens after acquisition of point mutations in the hemagglutinin cleavage site or gene segments from highly pathogenic H5N1 and H7N7 viruses.* Gischke, M., Winter, F., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

FLI Junior Scientist Symposium, 20 – 22 Sep. 2017, Braunschweig, Germany, Oral and Poster presentation by F. Winter. *Investigations of virulence determinants of highly pathogenic H10 avian influenza viruses.* Winter, F., Gischke, M., Veits, J., Mettenleiter, T.C., Abdelwhab, E.M.

27th Annual Meeting of the Society for Virology, 22 – 25 Mar. 2017, Marburg, Germany, Poster presentation by A.H. Salaheldin, *Novel mutations in the avian influenza H5N1 neuraminidase that modulate sialidase activity and virulence in mice with minimal impact on virus replication in cell culture and virulence or transmission in chickens.* Salaheldin, A.H., Hoffmann, D., Gischke, M., Veits, J., Hafez, H., Mettenleiter, T.C., Abdelwhab, E.M.

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