Aus dem Institut für molekulare Virologie und Zellbiologie
des Friedrich-Loeffler-Instituts
Bundesforschungsinstitut für Tiergesundheit
und
dem Institut für Veterinär-Biochemie
des Fachbereichs Veterinärmedizin der
Freien Universität Berlin

Virulence of Avian Influenza H7 Viruses in Birds: The Impact of the Hemagglutinin Polybasic Cleavage Site on the Virulence of a Recent German H7N7 Virus and Pathogenicity of European H7N7 and H7N1 Viruses in Ducks

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
David Scheibner
Tierarzt aus Stralsund

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Dedication

THIS WORK IS DEDICATED TO MY LOVELY FAMILY AND MY GIRLFRIEND.

THANKS FOR YOUR SUPPORT, PATIENCE AND MOTIVATION.

IN MEMORY OF MY FATHER AND MY GRANDPARENTS.

Content

List of figures and tables

Abbreviations

AA	Amino acid
AIV	Avian influenza virus
ATP	Adenosin triphosphat
cRNA	Complementary ribonucleic acid
C	Carboxy
COOH	Carboxyl group
CS	Cleavage site
E	Glutamic acid
ECE	Embryonated chicken eggs
EE	Early endosomes
ER	Endoplasmic reticulum
F	Phenylalanine
G	Glycine
H	Hydrogen
HA	Hemagglutinin
HACS	Hemagglutinin cleavage site
HP	Highly pathogenic
HPAIV	High pathogenic avian influenza virus
K	Lysine
L	Leucine
LE	Late endosomes
LP	Low pathogenic
LPAIV	Low pathogenic avian influenza virus
LP-poly	LP + polybasic cleavage site
M	Matrix protein
N	Nitrogen
NH_2	Amino group
mRNA	Messenger RNA
NA	Neuraminidase
NEP	Nuclear export protein
NP	Nucleoprotein
NS	Non-structural protein
O	Oxygen
PA	Polymerase acidic
P	Prolin
PB	Polymerase basic
R	Arginine
RIG-I	Retinoic acid inducible gene I
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT-PCR	Reverse-transcription polymerase chain reaction
SA	Sialic acid
T	Threonine
UK	United Kingdom
USA	Unites States of America
V	Valin
vRNA	Viral ribonucleic cid
WHO	World Health Organization

Chapter 1

General Introduction

1 Introduction

Avian influenza viruses (AIV) are members of the Influenza A genus in the family *Orthomyxoviridae*. They are enveloped pleomorphic viruses with a segmented genome composed of eight segments which encode for at least ten viral proteins. The surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), vary antigenically into 16 HA and 9 NA subtypes. Each AIV carries one HA and one NA with 144 possible combinations. Wild aquatic birds, particularly waterfowls, serve as natural reservoir for all AIV subtypes. Transmission of AIV from wild to domestic birds is a continuing threat particularly to farmed poultry as well as zoo and feral birds worldwide. Whereas all AIV subtypes are low pathogenic (LP) in birds causing no or mild clinical signs, some viruses of H5 and H7 subtypes can become highly pathogenic (HP) after circulation in gallinaceous birds (e.g. chickens and turkeys) causing up to 100% mortality within a few days. Mutations in the HA, particularly in the HA cleavage site (CS), are major virulence determinants of high pathogenic AIV (HPAIV), although mutations in other gene segments can modulate replication, virulence and bird-to-bird transmission.

H7N7 is the most abundant H7 subtype and is circulating in wild birds in Europe. The isolation of low pathogenic AIV (LPAIV) H7N7 from domesticated poultry has been frequently reported. Some LPAIV H7N7 shifted to HPAIV H7N7 causing significant economic losses due to high mortality or culling of infected birds. In 2015, layer chickens in two farms in Emsland, in the North West of Germany, became infected with H7N7. An LPAIV was detected in birds in the first farm, while both LP and HPAIV were isolated in chickens in the second farm. The LPAIV was the putative precursor of the HPAIV H7N7. Such a natural pair of viruses is very important for understanding the genetic determinants for the evolution of HPAIV from LPAIV.

In this dissertation the impact of the hemagglutinin cleavage site (HACS) (chapter 2) on virulence of the current German H7N7 isolates was determined in chickens, turkeys and ducks. Furthermore, the virulence of historic and recent H7 viruses in Pekin and Muscovy ducks was assessed (chapter 3). These findings increase our understanding of the evolution and pathogenesis of HPAIV H7 in different bird species, which is essential for risk assessment and control of AIV H7N7.

2 Review of literature

2. 1 Avian influenza virus

2.1.1 Virus taxonomy and structure

AIV belong to the family *Orthomyxoviridae*. The members of this family are enveloped viruses with a single-stranded, negative-sense, segmented ribonucleic acid (RNA) genome of six to eight gene segments. To date, this family includes seven genera: Influenza A, B, C, D, Thogotovirus, Quaranjavirus and Infectious Salmon Anemia (Isavirus) virus (Viruses and ICTV, 2018). AIVs belong to the genus Influenza A virus which are pleiomorphic in shape but mostly spherical (Samji, 2009). They can be allocated into different genetic lineages like the North American, the Australian and the Eurasian lineages. The two major surface glycoproteins HA and NA are differentiated by serological tests like hemagglutination and NA inhibition into 16 HA and 9 NA subtypes. Each AIV carries one HA and one NA subtype with 144 potential HxNx combinations. Also, phylogenetic and sequence tools are useful for the differentiation of AIV subtypes (Fouchier et al., 2005). HAs can be divided into two different phylogroups: group 1 contains H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16, and group 2 includes H3, H4, H7, H10, H14 and H15. Likewise, the NA is differentiated into group 1 including N1, N4, N5 and N8, and group 2 with N2, N3, N6, N7 and N9 (Gamblin and Skehel, 2010). Furthermore, different HA clades and subclades can be distinguished based on the genetic variations within each subtype (Mostafa et al., 2016; Stucker et al., 2015). The nomenclature of influenza virus has been recommended by the World Health Organization (WHO) (WHO, 1980) in the first influenza meeting in early 1980s. Therefore, the name should contain the influenza type, host where the virus was isolated from (except for human isolates), geographical origin, strain number, year of isolation and subtype, as avian isolate A/chicken/Germany/AR915/2015 (H7N7) or human isolate A/Puerto Rico/1/1934 (H1N1).

AIVs contain eight gene segments (segments 1 to 8) of together about 13,500 nucleotides, coding for at least ten viral proteins (Fig. 1) (Bouvier and Palese, 2008; Ghedin et al., 2005). The three largest segments 1, 2 and 3 encode for the polymerase basic 2 (PB2), polymerase basic 1 (PB1) and polymerase acidic (PA), respectively, which are components of the viral polymerase. Together with the nucleoprotein (NP), encoded by the 5th segment and the viral RNA, the polymerase proteins form the viral ribonucleoprotein complex (RNP). The RNP is connected to the envelope of the virus derived from the host cell membrane via the Matrix protein 1 (M1), which is located beneath the envelope and is one of the two proteins encoded by the 7th segment (Bouvier and Palese, 2008). The second protein encoded by this segment is the Matrix protein M2 embedded in the virus envelope with a short ectodomain. It acts as an ion-channel, which plays an important role in uncoating of the virus genome into the host cells. Segment eight is the smallest gene segment. It is also dicistronic and encodes for the non-

structural protein (NS) 1, which interferes with the host innate immune response, and the nuclear export protein NEP (or NS2), which is essential for the transport of newly formed viral genome segments from the nucleus to the cytoplasm followed by budding of the progeny virions (Selman et al., 2012). The HA (encoded by the 4th segment) mediates virus entry into the host cell and fusion with the host cell membrane. The NA (encoded by the 6th segment) enables virus release from the infected cells. On most viruses a ratio of 4:1 of HA to NA can be found (Webster et al., 1968). Since 2001 further viral proteins have been identified in influenza A viruses like PB2-S1 (Yamayoshi et al., 2016), PB1-N40 (Wise et al., 2009), PA-N155, PA-N182 (Muramoto et al., 2013), M42 (Wise et al., 2012) and NS3 (Selman et al., 2012) which are non-essential. Nevertheless, PB1-F2 and PA-X are proteins which are also expressed by the second and the third segment, respectively and play a role in apoptosis and immune response for example (Chen et al., 2001; Jagger et al., 2012).

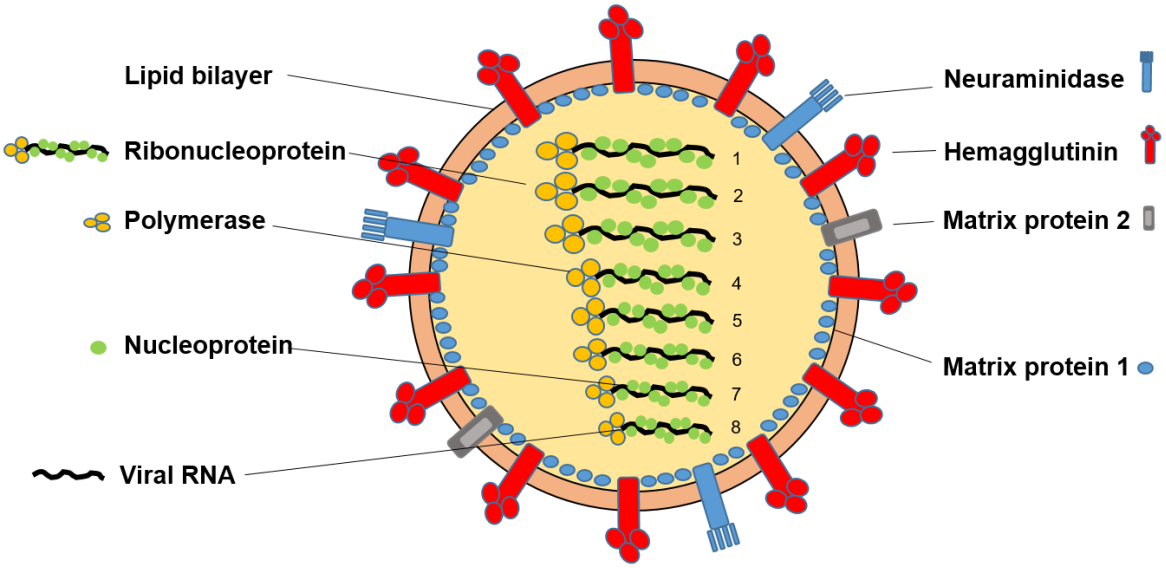

Figure 1: Structure of an influenza virion. The virion has an envelope formed by a double lipid bilayer membrane derived from the host cell. HA, NA and M2 are embedded in the viral envelope by transmembrane domains. The eight genome segments are stabilized by the nucleoprotein and carry the viral polymerase complex forming the RNPs. The M1 lays under the membrane and interacts with the RNPs. Modified from Racaniello (2009)

2.1.2 Virus replication

Unlike other RNA viruses, the replication of AIV takes place inside the nucleus of infected cells. To start the replication cycle, the virus attaches to the host cell receptor via the HA (Figure 2). The affinity of HA to different sialic acid (SA) moieties predetermines host specificity. Avian species like waterfowl express α-2,3-SA receptors in the respiratory and the intestinal tracts, while human tissues for example express mostly α-2,6-SA receptors in the upper respiratory

tract and less frequently α-2,3-SA receptors in the lungs (Byrd-Leotis et al., 2017; Connor et al., 1994; de Graaf and Fouchier, 2014; Webster et al., 1992). These SAs are nitrogen (N) or Oxygen (O) substituted derivatives of neuraminic acid and widely expressed on the surfaces of all cells in all animals (and some bacteria infecting animals) (Varki, 2008). These biomolecules play important roles in the host metabolism as they regulate fluid and ion uptake into the cell. They are also important for the host immune response and brain evolution (Du et al., 2009). Once the virus has attached to the SA, it enters into the cell, mostly via clathrin-mediated endocytosis or macropinocytosis (De Conto et al., 2011; Rust et al., 2004). This first step of virus uptake is called internalization and takes only a few minutes. Subsequently the virus is transported via early endosomes (EE) to the perinuclear region of the cell (Lakadamyali et al., 2003). Mediated by an adenosine triphosphate (ATP) dependent proton pump the EE is acidified (from pH 6.0 to 4.8) and turns into a late endosome (LE). In this phase the ion-channel M2 plays an important role (Wharton et al., 1994). By proton transport from the endosome into the virion, conformational changes in the HA and the M1 proteins are induced. Lowering the pH leads to the fusion of the viral envelope with the endosomal membrane which is mediated by the HA stem bringing both membranes, viral and endosomal, in close proximity (Bullough et al., 1994; Chen et al., 1999) resulting in formation of a fusion pore (Tatulian et al., 1995). To release the viral RNPs, M1 has to detach from the viral membrane and subsequently the NP dissociates from M1 and the RNP complex is released into the cytosol (Bui et al., 1996; Fontana et al., 2012), a step known as uncoating in which several viral and host factors are involved and interact (Banerjee et al., 2014).

The viral RNA is stabilized by the NP via positively charged amino acids (AA) and carries the viral polymerase which is arranged in the following order: amino group (NH_2)-PA-PB1-PB2-carboxyl group (COOH). Interaction between the polymerase complex and the NP has been described (Eisfeld et al., 2015; Pflug et al., 2017; Stevaert and Naesens, 2016). The transport into the nucleus is mediated by nuclear localization signals located in the NP and cellular transport proteins like importin-α. Once the viral genome, wrapped by the NP, has been imported into the nucleus, two distinct steps start in parallel. The negative-sense viral RNA (vRNA) is transcribed into positive-sense complementary RNA (cRNA) which subsequently acts as a template for the generation of the vRNA segments of the progeny virions (Engelhardt et al., 2005; Lukarska et al., 2017). The new RNA is stabilized by binding with the NP. In parallel, transcription of the vRNA into messenger RNA (mRNA) takes place. Both steps are mediated by the viral polymerase. PB2 mediates the cap-snatching, “stealing” of primers from cell mRNAs to enable translation by the host machinery (Dias et al., 2009). PA acts as an endonuclease and PB1 is the actual polymerase elongating the RNA. Conserved 5´ ends of the influenza segments composed of several uracil nucleotides result, after transcription, in a

poly-Adenylate-tail within the mRNA. The viral NEP and NP are involved in the transport of the new RNPs to the endoplasmic reticulum (ER) (Eisfeld et al., 2015).

In the ER the viral proteins are modified. Since all proteins are built as monomers first they have to be arranged as di-, tri- or tetramers depending on their function. The connection is enabled by intrasubunit disulfide bonds between certain cysteine residues. HA forms a homotrimer (Kemble et al., 1992), NA (Air, 2012) and M2 (Holsinger and Lamb, 1991) form homotetramers, NS1 is a homodimer (Marc, 2014) and NP forms trimers or even larger oligomers (Ng et al., 2008). Moreover, glycosylation of the surface proteins HA and NA is important for their function. Number and distribution of glycosylation sites in the HA and NA are strain dependent (Hutchinson et al., 2012). Further modifications include phosphorylation of all AIV structural proteins, in addition to the NS1 (Hutchinson et al., 2010) and palmitoylation which so far has only been reported for HA and M2 (Naeve and Williams, 1990).

Budding of the virus takes place in close vicinity to the plasma membrane in a so called “budozone”. The three membrane embedded proteins HA, NA and M2 carry apical sorting signals which guide them to the plasma membrane (Schmitt and Lamb, 2005). Virus assembly requires M1 protein interaction with the cytoplasmic tails of HA, NA and M2 and with the RNP-NEP complex (Noton et al., 2007; Schmitt and Lamb, 2005). Each RNA segment carries specific packaging signals, located at both ends of the viral RNA. They are essential for packaging of all eight gene segments into the virion (Fujii et al., 2003). Mutations in the packaging signals lead to false packaging. The eight segments are organized in a 1+7-arrangement, where one segment is located in the center and is surrounded by the other seven segments (Eisfeld et al., 2015; Fournier et al., 2012). The HA of the newly formed virions is attached to the sialic acid moieties on the cell membrane. Therefore, NA is necessary to remove sialic acid bonds and release the progeny viruses preventing aggregation of virions on the cell surface (Colman, 1994). Like the HA, the NA prefers particular SA receptors (Calder et al., 2010). Thus, the HA-NA balance plays an important role for virus replication in the host cells (Wagner et al., 2002).

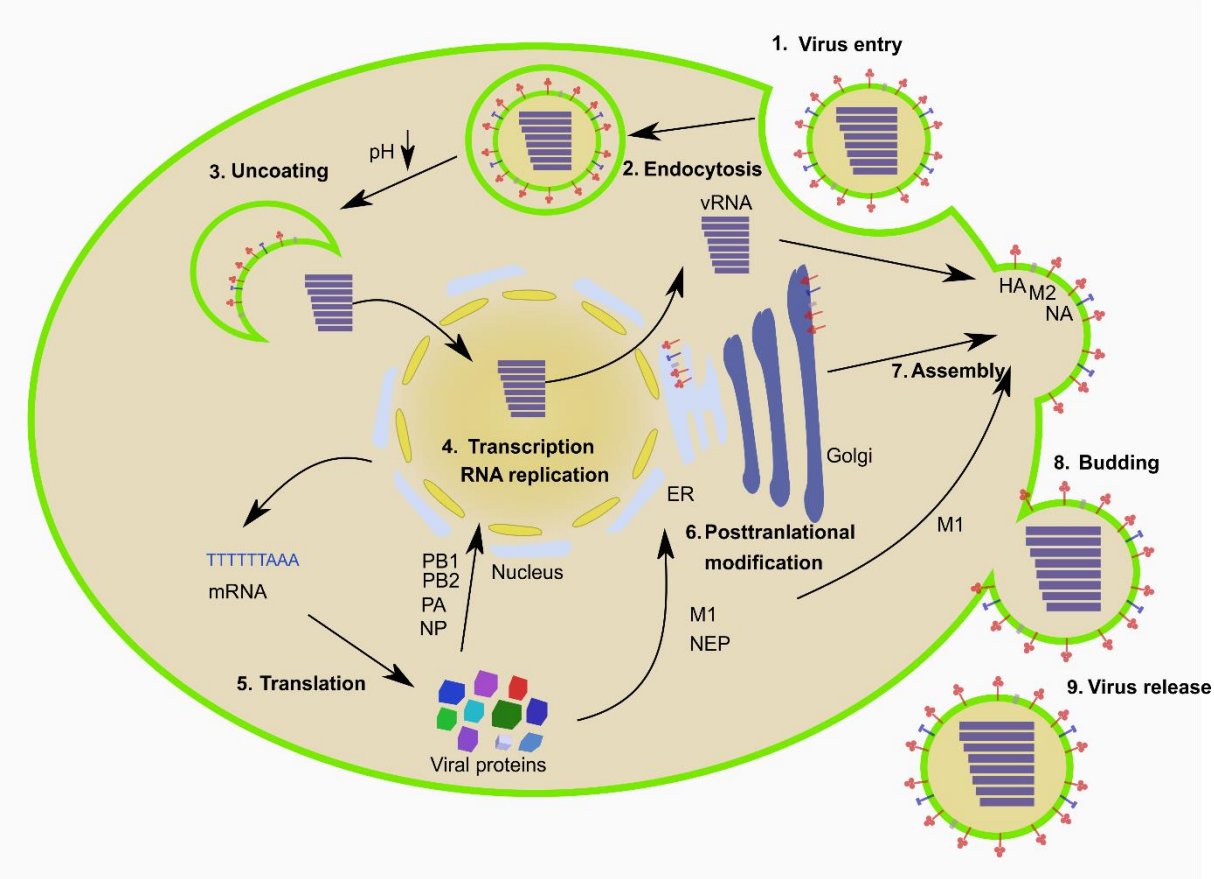

Figure 2: Influenza A virus replication cycle. (modified from Arias et al. (2009)) After attachment of the HA to the host cell sialic-acid receptors, virus entry (**1**) is mediated by endocytosis (**2**). A decrease of the endosomal pH activates fusion of the viral and endosomal membrane which leads to release of the viral genome or uncoating (**3**). After the transcription into mRNA (**4**) and viral RNA replication (**5**) newly translated proteins (**6**) are modified in the endoplasmic reticulum and Golgi apparatus (**7**). The assembly of viral RNA and the mature proteins takes place at the plasma membrane (**8**), followed by virus budding (**9**). Finally, NA catalyzes the dissociation between HA and sialic-acids and leads to virus release (**10**).

2.1.3 Proteolytic activation

Virus replication is dependent on activation by host proteases. The HA protein, which enables attachment and fusion with the endosomal membrane and thus RNP release, is generated as a fusion-inactive molecule HA0 (Steinhauer, 1999). Depending on the structure of the HACS different host proteases can cleave HA0 into HA1 and HA2 and predetermine host and tissue tropism of the virus (Klenk and Garten, 1994). LPAIV exhibit a monobasic HACS motif **arginine**/glycine leucine phenylalanine (**R**/GLF) recognized by trypsin-like proteases (Klenk and Garten, 1994). These proteases are known to be restricted to the respiratory and the intestinal tracts. All HA subtypes carrying a monobasic CS cause local infections (Bosch et al., 1981). Conversely, HPAIV H5 and H7 can contain a polybasic HACS motif like **R-X-lysine (K)/R-R**/GLF (X is a random residue), which is activated by ubiquitous subtilisin-like proteases (Steinhauer, 1999; Stieneke-Grober et al., 1992). Since these proteases occur in many cells

and organs, HPAIV cause systemic infections, multiorgan dysfunction and up to 100% mortality. While LPAIV are activated by exogenous proteases, HPAIV are activated by exogenous and endogenous furin-like proteases located at the plasma membrane or in the ER (Stieneke-Grober et al., 1992).

2.1.4 Virus evolution

Evolution of AIV in nature occurs through three genetic mechanisms: antigenic drift, antigenic shift and recombination. Antigenic drift is the gradual accumulation of point mutations in different proteins. These mutations are frequently acquired due to the error-prone nature of the viral RNA-dependent RNA-polymerase (Shao et al., 2017). Spontaneous mutations introduced by the viral polymerase and the absence of exonuclease activity lead to a very high mutation rate (Ahlquist, 2002; Chen and Holmes, 2006). Since HA and NA are the main immunogenic proteins, mutations which change the epitopes of these proteins can lead to immune escape. HA has five epitopes (A-E) where changes in the two major epitopes, epitope A and epitope B, have the highest impact on immune escape. This plays an important role for vaccination efficiencies (Gamblin et al., 2004). The NA protein contains three immunogenic epitopes (A, B and C). However, mutations leading to immune escape are less frequent than in the HA (Munoz and Deem, 2005). Furthermore, single mutations in the HA receptor binding site can influence host specificity by the variation of sialic-acid binding affinity (Gabriel et al., 2005). Mutations in the polymerase complex can also lead to a shift from avian to mammalian species by increasing replication in the new host (Dortmans et al., 2013). Mutation also plays an important role in the acquisition of a polybasic cleavage site by HPAIV, mostly through a spontaneous duplication of a purine-triplet as the most common way for amino acid insertions. Nevertheless, it could also be shown that insertions can occur without nucleotide repetition (Perdue et al., 2003).

The second mechanism is called genetic shift which means the exchange of whole gene segments during infection of a host cell with different parental viruses (Reid and Taubenberger, 2003). These viruses can be highly fatal in naïve human and poultry populations without prior exposure to the new reassortant virus. The influenza pandemics in 1918/1919 and 2009 for example were caused by human influenza viruses carrying gene segments from avian and/or porcine influenza viruses. The latter species plays an important role as a mixing vessel because it expresses the avian-type and mammalian-type of SA receptors (Morens and Taubenberger, 2011; Morens et al., 2010).

The third mechanism leading to new virus variants is recombination. Parts of influenza virus gene segments or even RNA fragments from the host are exchanged with parts of other gene segments (Chare et al., 2003). In H7N3 viruses isolated in Canada (2004) and Chile (2002)

this mechanism led to unusual polybasic cleavage sites by recombination with the M and the NP gene, respectively (Pasick et al., 2005; Suarez et al., 2004).

2.2 Pathogenicity of AIV in different host species

2.2.1 Domestic Poultry

While all bird species can be affected by AIV (Alexander, 2000), gallinaceous birds like chickens and turkeys are known to be especially susceptible to AIV infections. Generally, LPAIV cause asymptomatic or mild infection, which is exacerbated by concurrent viral or bacterial infections. Infection of the upper respiratory tract with LPAIV is associated mainly with rhinitis, sinusitis, swollen infraorbital sinuses and nasal discharge, particularly in turkeys (Pantin-Jackwood and Swayne, 2009). Furthermore, nephrosis or nephritis is common in layer chickens, while pancreatitis has been reported in turkeys (Pantin-Jackwood and Swayne, 2009). Turkeys can be easily infected experimentally with low virus doses (Pillai et al., 2010). Also, field outbreaks like the H7N1 epidemic from 1999-2000 in Italy revealed a high susceptibility of turkeys with age-dependent mortality ranging from 5 to 97%. After infection with LPAIV, mortality in young turkeys (up to 40 weeks) is higher than in older birds. Postmortem examination demonstrated severe pathological findings like hemorrhages in the pancreas or fibrinous casts in sinuses and trachea leading to death by asphyxia. Breeder turkeys showed decreased egg production and weight loss, while breeder chicken and broilers exhibited only mild clinical signs, if any (Capua et al., 2000a). HPAIV infections are usually severe in both, chickens and turkeys (Alexander et al., 1986; Carnaccini et al., 2019; Jeong et al., 2009). Clinical signs like hemorrhages on the legs, cyanosis and necrosis of wattles and/or combs are very prominent in chickens (Figure 3). Also central nervous disorders can be observed in more slowly replicating viruses in chickens but seem to be more common in turkeys (Figure 3) (Pantin-Jackwood et al., 2017b). Interestingly, a chicken-origin virus was more virulent in chickens and a turkey-origin virus showed higher virulence in turkeys which indicates variations in the susceptibility of chickens and turkeys to some HPAIV (Alexander et al., 1986; Pantin-Jackwood and Swayne, 2009).

Waterfowl is less affected by AIV than chickens and turkeys. Susceptibility of domestic ducks to H5 viruses has been extensively studied. Several factors modulated clinical signs and mortality in ducks including species, age and inoculation route (Pantin-Jackwood et al., 2013; Pantin-Jackwood et al., 2012). The commonly used breeds for experimental infections are Muscovy, Pekin and Mallard ducks. Infection with LPAIV causes no clinical signs in ducks and spread is very limited (Franca et al., 2012; Jourdain et al., 2010). Pantin-Jackwood et al. (2013) reported higher susceptibility of Muscovy ducks to HP H5N1 infections compared to other duck species. They further showed that all tested duck species including Pekin and Mallard can be

infected via intranasal, intraocular and intracloacal inoculation routes (Pantin-Jackwood et al., 2013). Other studies showed differences in virulence in Pekin ducks after intramuscular or intranasal inoculation (Grund et al., 2018). Interestingly, age-dependent susceptibility is highly prominent in ducks. The infection with HPAIV H5N1 resulted in more severe clinical signs in ducklings up to two-weeks-old compared to ducks older than four weeks (Pantin-Jackwood and Swayne, 2007) which was associated with a difference in the immune response (Pantin-Jackwood et al., 2012; Wei et al., 2013). Viral RNA sensing in host cells and subsequent interferon response is mediated by several pathways including the retinoic acid inducible gene I (RIG-I) (Pichlmair et al., 2006; Schlee et al., 2009; Yoneyama et al., 2004). The resistance of ducks to AIV infection is associated with upregulation of RIG-I. It is worth mentioning that chickens and turkeys lack this gene (Barber et al., 2010; Magor et al., 2013). Nevertheless, although rarely, some HPAIV H5 caused clinical signs including nervous signs (e.g. torticollis and opisthotonus) and mortality (Ellis et al., 2004; Grund et al., 2018; Kishida et al., 2005; Lee et al., 2005).

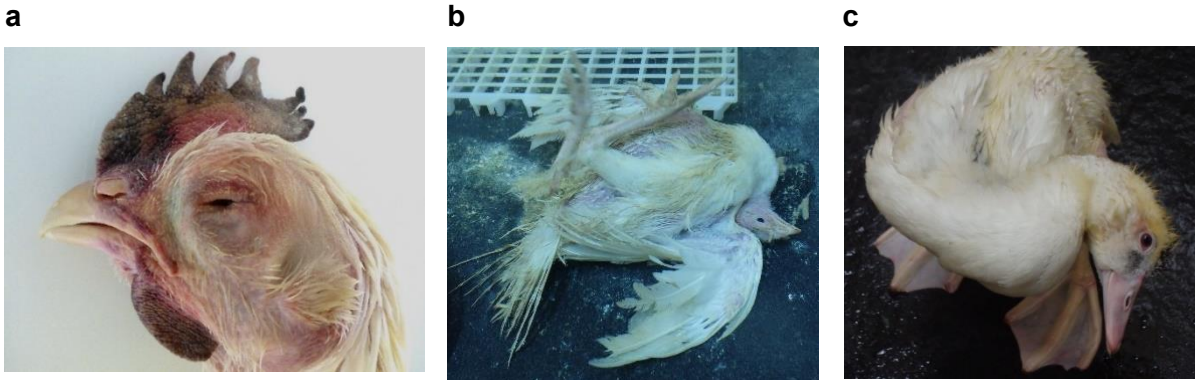

Figure 3: Clinical signs after HPAIV infection. Chicken showing cyanosis and necrosis of the comb and the wattles (**a**), turkey with nervous signs like seizures and spasms (**b**) and duck with torticollis (**c**). (Foto: Reiner Ulrich, Abteilung für Tierhaltung und Biosicherheit, David Scheibner, Friedrich-Loeffler Institut)

2.2.2 Wild birds

Wild birds are the reservoir of AIVs, where the evolution of AIV is thought to be in an equilibrium. Aquatic birds like *Anseriformes* (e.g. ducks and geese) and *Charadriiformes* (e.g. gulls or shorebirds) are the most important reservoir for AIV (Alexander, 2000; Kawaoka et al., 1988; Slemons et al., 1974). Efficient replication in epithelial cells of the gastrointestinal tract lead to virus shedding in the feaces at high titers for long periods (Hinshaw et al., 1980; Webster et al., 1978). Thus, the feacal-oral route is very common for transmission in wild aquatic birds (Abdelwhab el et al., 2016; Alexander, 2007; Hinshaw et al., 1979). Transmission of AIV from wild birds to poultry has been frequently reported via direct contact in poultry premises or live bird markets or by the consumption of contaminated water or feed (Alexander,

2007; Kung et al., 2003; Sims et al., 2016; Suarez et al., 1999). Wild ducks excrete a high concentration of the virus mainly via the cloaca, estimated to be around $4.58x10^8$ mean egg infection doses per gram (Webster et al., 1978). Although AIV induced no clinical signs in wild birds, in few occasions, high mortality was observed in wild birds caused by HPAIV H5Nx viruses. The first HPAIV outbreak in wild birds was an HPAIV H5N3 in common terns in South Africa in 1961 (Becker, 1966). Since 2002 frequent cases of H5N1 infections have been reported leading to mortality in ducks (Ellis et al., 2004). In 2016, HPAIV H5N8 caused nervous signs and mortality in Pekin ducks (Grund et al., 2018).

2.2.3 Mammals

AIV infect a wide range of mammals including humans. Pigs are known to be a mixing vessel for influenza viruses since they express both avian-type and mammalian-type receptors resulting in devastating pandemics in 2009 and presumably in 1918/1919 (Morens and Taubenberger, 2011; Morens et al., 2010). Recent infection of horses is mostly limited to H3N8 viruses (Guo et al., 1992; Guo et al., 1995). LPAIV and HPAIV were isolated from cats, dogs, mink, stone martins, leopards and tigers (Englund et al., 1986; Klingeborn et al., 1985; Kuiken et al., 2004; Parrish and Voorhees, 2019; Thanawongnuwech et al., 2005; Xue et al., 2018). Furthermore, AIV was isolated from marine mammals (e.g. seals and whales) which had been infected sometimes with fatal consequences (Callan et al., 1995; Hinshaw et al., 1986; Hinshaw et al., 1984; Nielsen et al., 2001). Morbidity and mortality were reported in seals infected with LPAIV H3N8 in 2011 (Anthony et al., 2012; Karlsson et al., 2014) and H10N7 in 2014 (Bodewes et al., 2016). In addition, mice, ferrets and guinea pigs are susceptible to AIV and are usually used to study the zoonotic potential of AIV (Nagy et al., 2017; Sutton, 2018; Sutton et al., 2014).

2.2.4 Humans

Several avian influenza viruses, including H7 subtypes, have high zoonotic potential (Mostafa et al., 2018). First detections of avian influenza viruses in humans were reported in 1959 in the USA followed by cases in Australia in 1977 and again USA in 1978-1979 which were all caused by H7N7 (DeLay et al., 1967; Kalthoff et al., 2010; Webster et al., 1992; Webster et al., 1981). In 2003, an epidemic of H7N7 in poultry in the Netherlands led to 89 human infections. Although most of them were self-limiting or caused mild conjunctivitis, one veterinarian died from the infection (Belser et al., 2009a; Koopmans et al., 2004; van Kolfschooten, 2003). In addition, HPAIV H7N7 was diagnosed in three poultry workers in Italy in 2013 (Puzelli et al., 2014). Moreover, several countries reported infections of poultry workers or occupational groups by different H7N2 in the United Kingdom (UK) and the Unites States of America (USA)

or H7N3 in Canada, Italy, Mexico and the UK (Nguyen-Van-Tam et al., 2006; Ostrowsky et al., 2012; Skowronski et al., 2006; Tweed et al., 2004). H7 antibodies were detected in poultry workers or occupational groups for example in Italy (Puzelli et al., 2005). More recently an H7N4 affected a 68 year-old woman (Tong et al., 2018). From 2013 to date, H7N9 led to about 1568 confirmed human cases with a fatality rate of 39% in Asia marking the largest human infections by bird-origin viruses (FAO, 2019b; Lam et al., 2013).

2.3 Genetic determinants for virulence in and adaptation of AIV to poultry

2.3.1 Virulence determinants in chickens

2.3.1.1 Hemagglutinin: HA cleavage site, its vicinity and head domain

HA is a class 1 transmembrane glycoprotein. It is the most abundant viral surface protein and plays an important role in virulence, immunogenicity and host adaptation of AIV. The HA0, the fusion-inactive precursor, is a trimeric protein which is unable to mediate a membrane fusion with the host membrane to start the viral replication cycle. HA has to become activated by host proteases by cleavage into HA1 and HA2 subunits at the arginine and glycine bond (R-X-K/R-R/G in case of HPAIV or R/G in case of LPAIV) (Stieneke-Grober et al., 1992). The HA has a globular head domain formed only by HA1, and a stem, which is formed by HA1 and HA2. HA1 mediates attachment to the cell with the receptor binding residues in the globular head domain. It also carries the five antigenic epitopes A-E. HA2 contains the hydrophobic fusion peptide which enables fusion of viral and endosomal membranes in early stages of the virus replication cycle (Durrer et al., 1996). HA2 is anchored with the transmembrane domain in the virus envelope and carries a cytoplasmic tail at the NH_2-terminus (Isin et al., 2002; Laver, 1971; Wiley and Skehel, 1987). Once HA0 is cleaved, the fusion peptide interacts with the endosomal membrane for release of the viral genome into the host cell (Bullough et al., 1994; Chen et al., 1999; Durrer et al., 1996).

The transformation of the monobasic HACS of LPAIV to a polybasic HACS usually results in the evolution of HPAIV H5 and H7 viruses. Several studies have shown that the polybasic HACS can be sufficient for high virulence of HPAIVs in chickens (Abdelwhab et al., 2016; Munster et al., 2010; Veits et al., 2012) It is therefore considered the main virulence determinant in poultry (Bosch et al., 1981; Klenk and Garten, 1994; Stieneke-Grober et al., 1992). However, some viruses with polybasic HACS are avirulent in chicken (Londt et al., 2007). Likewise, using reverse genetics insertion of a polybasic HACS to H5 or H7 AIV in addition to several non-H5/H7 viruses did not result in high virulence in chickens (Bogs et al., 2010; Stech et al., 2009).

Mutations in the vicinity of the HACS play an important role in virulence via different mechanisms. For example, glycosylation sites close to the HACS of the 1983 Pennsylvanian HPAIV H5N2 sterically hindered the access of proteases to the polybasic CS and limited the spread to the respiratory and intestinal tracts of chickens resembling LPAIV. Deglycosylation or increasing the number of basic amino acids in the HACS resulted in trypsin-independent activation of the virus and shift to high virulence (Kawaoka and Webster, 1989). Moreover, in addition to the polybasic HACS, three mutations in the HA2 of an HPAIV H7N1 were important for full virulence and transmission in chickens. These mutations were important for pH fusion, stability and replication of the virus (Abdelwhab el et al., 2015). Furthermore, the HA1 head domain is known to carry a variable number of N-glycosylation sites compared to the stem region where glycosylation is more conserved (Wang et al., 2009). The glycosylation of the HA head domain and the truncation of the NA stalk domain acted synergistically to exhibit high virulence in chickens (Li et al., 2011; Matsuoka et al., 2009). In addition, other mutations in the HA1 domain were found to influence cleavability or fusion activity of the HA of H5N2 and H5N1 viruses and therefore regulated virulence (Deshpande et al., 1987; Wessels et al., 2018).

2.3.1.2 Neuraminidase: NA stalk deletion and reduction in NA activity

The NA is the second most abundant viral surface glycoprotein. It plays an important role in virulence, tropism and host adaptation (Li et al., 2011; Varghese et al., 1983). The neuraminidase is a homotetramer and each monomer contains around 470 amino acids. Four distinct structural and functional domains can be distinguished: the cytoplasmic tail, the transmembrane domain, the stalk-domain and the catalytic head-domain (McAuley et al., 2019). The NH_2-terminal cytoplasmic tail interacts with M1 and thus mediates virus budding. This domain is highly conserved among the Influenza A viruses due to its important impact on virus replication (Blok and Air, 1982; Enami and Enami, 1996). The transmembrane domain consists of hydrophobic AA and interacts with cell and viral membrane (Blok and Air, 1982). Thus, it is essential for NA trafficking to the plasma membrane (Barman and Nayak, 2000). The stalk domain of NA has conserved cysteine residues and N-glycosylation amino acids, which stabilize the tetrameric structure of the protein (Blok and Air, 1982). The NA stalk domain varies in length due to deletions of 1 to 36 amino acids in NA N1, N2, N3, N5, N6 and N7 subtypes. A deletion of 20 amino acids was observed frequently in the goose/Guangdong H5N1 lineage since 2000s in domestic birds compared to wild bird isolates (Li et al., 2011), which is probably advantageous for adaptation of AIV from waterfowl to domestic poultry (Matsuoka et al., 2009). Stech et al. (2015) showed that in addition to the polybasic HACS, deletion of the NA stalk domain was important for virulence of an HPAIV H5N1 in chickens. Moreover, it has also been shown that some influenza viruses with shorter NA stalk-domains replicated less efficiently in mice and/or chicken eggs. Conversely, the pandemic H1N1 virus

from 2009 with shorter NA stalk exhibited high virulence in mice and ferrets (Park et al., 2017). This deletion in the NA stalk domain reduced the NA activity of the virus. Reduction of NA length has probably decreased the access of the NA to sialic acids. This is supported by simultaneous reduction in binding affinity of the HA to sialic acid which lower the dependency of the virus on NA alone (Baigent et al., 1999; Baigent and McCauley, 2001; Nguyen et al., 2013; Wagner et al., 2000). Another explanation is that changes in the stalk domain may induce conformational changes in the catalytic head domain influencing sialidase activity (Durrant et al., 2016). Apart from the stalk domain, mutations in the NA head domain also influenced immune-escape and susceptibility to antivirals (McKimm-Breschkin, 2013; Yasuhara et al., 2019).

2.3.1.3 Other gene segments

Several studies have shown the importance of other gene segments in virulence of influenza viruses, mainly in chickens and to lesser extent in ducks. The NS-segment encodes for two proteins: NS1 and NEP. The latter is mainly mediating the transport of the progeny viral genome into the cytoplasm, while NS1 has several functions and can play a crucial role in virulence of AIV. NS1 is composed of a RNA-binding domain which binds to different types of RNA and the effector domain which contains nuclear export signals and interacts with several host factors like the RIG-I and different kinases (Abdelwhab et al., 2013). Importantly, the variation in the COOH-terminal domain of the NS1 due to deletion or extension has been comprehensively analyzed (Abdelwhab el et al., 2016). In chickens, the NS was not essential for the virulence of the Italian H7N1, although truncation in the COOH-terminal domain accompanying the shift of HP to LP was essential for virus excretion (Abdelwhab el et al., 2016). Moreover, it has been shown that H5N1 virus with a single mutation at position 149 within the NS1, in addition to the polybasic HACS, was highly virulent in chickens due to increased interferon antagonism (Li et al., 2006). Furthermore, the NS segment from an H5N1 increased virulence of H7 viruses in embryonated chicken and turkey eggs (Petersen et al., 2013). The contribution of aspartic acid 44 asparagine in the M2, in addition to the polybasic HACS, to higher virulence of an H5N3 in chickens was reported (Fujimoto et al., 2015)

2.3.2 Virulence determinants in ducks and turkeys

Very little is known about virulence determinants of AIV in turkeys and ducks compared to chickens. The polybasic CS alone seems not to play an important role as virulence determinant in ducks since they are mostly resistant against HP infections (Kuchipudi et al., 2014; Pantin-Jackwood et al., 2017a; Slomka et al., 2018). Thus, mutations in the HA or other gene segments serve as virulence determinants (Hu et al., 2013). The PB1-F2 encoded by the

second segment is well conserved among AIV. It is a non-structural pro-apoptotic peptide which disrupts the mitochondrial membrane potential especially in monocytes (Chanturiya et al., 2004; Chen et al., 2001). Deletion of the PB1-F2 decreased the virulence of an HPAIV H5N1 in ducks (Schmolke et al., 2011). In another study, tyrosine 436 histidine in PB1 and threonine 515 alanine in PA reduced pathogenicity of HP H5N1 in ducks after oculonasal inoculation (Hulse-Post et al., 2007). The importance of PA as a virulence determinant for H5N1 viruses in ducks has further been reported in a study by Song et al. (2011). Two mutations within the PA increased virulence in ducks, polymerase activity and PA-PB1 accumulation in duck embryo fibroblasts (Song et al., 2011). Hu et al. (2013) showed that the impact of PA on H5N1 virulence in ducks was even higher than the HA. Three mutations in the PA increased virulence, virus replication, polymerase activity and nuclear accumulation (Hu et al., 2013). Deletion of PA-X expressed by the third segment increased virus replication and polymerase activity of an H5N1 *in vitro* and *in vivo* in chickens, ducks and mice (Hu et al., 2015). Two HPAIV H5N1 isolated in Japan showed differences in their PB1 and M1 proteins. One virus was avirulent in ducks, while the second one was highly pathogenic. Interestingly, only one mutation at position 43 within the M1 protein increased virulence of the virus in ducks (Nao et al., 2015). Soubies et al. (2013) showed that truncations of the NS 1 C-terminus of an H7N1 virus modulated virulence in chickens but not in ducks. In addition, reassortment of LPAIV H5N1 with NS1 from HPAIV did not significantly alter the virulence of LPAIV in ducks indicating the importance of other gene segments for virulence in this species (Sarmento et al., 2010).

2.3.3 Vaccines and antivirals against AIV

In humans, combinations of influenza A (i.e. H1N1 and H3N2) and influenza B are available as vaccine strains but have to be adjusted every season due to mutations and frequent changes of the circulating viruses (Schotsaert and Garcia-Sastre, 2014). In poultry, since 1994-1995 vaccines against AIVs are used in some countries to mitigate the socioeconomic impact of AIV on the poultry industry (Capua and Marangon, 2007). Several inactivated monovalent or bivalent AIV vaccines are currently used in poultry in several countries. In addition, viral-vectored vaccines containing HA and sometimes NA were produced using fowl-pox virus, Newcastle disease virus, herpesvirus of turkeys, infectious laryngotracheitis virus, alphavirus and Adenovirus as backbone vectors (de Vries and Rimmelzwaan, 2016). Although these vaccines are able to reduce mortality, morbidity, virus excretion, bird-to-bird transmission and tissue tropism, annual or biennial update of these vaccines has been recommended to confer full protection against the immune escape mutants (Abdelwhab et al., 2014). These antigenic-drift variants evolve due to the immune pressure induced by vaccination of poultry,

resembling the seasonal influenza viruses in humans. They possess several point mutations in the immunogenic epitopes in the head domain of the HA (Abente et al., 2016).

There are two main categories of anti-influenza drugs. The M2-inhibitors, adamantanes, block the influx of protons into the virion in early stages of replication cycle and thus, prevent the release of the viral genome into the host cell. Representatives of this group are rimantadine and amantadine hydrochloride (Kato and Eggers, 1969). In humans, NA inhibitors including oseltamivir, zanamivir, peramivir and lanamivir are effectively used to prevent or treat influenza infections (Allen et al., 2006). However, resistance against both groups of antivirals has been frequently reported in both poultry and humans (Hurt et al., 2012). The resistant strains have higher biological fitness than the sensitive parents (Dong et al., 2015). Furthermore, favipiravir, a polymerase blocking drug, is available in Japan (Hussain et al., 2017). Chemotherapeutics, herbs and alternative medicine are also used in poultry and humans (Abdelwhab and Hafez, 2012; Boltz et al., 2010). Nevertheless, chemotherapeutics are not recommended to be used in poultry, to avoid emergence and transmission of resistant strains to humans.

2.4 Significance of AIV H7 infections in birds and humans

2.4.1 History and subtypes

H7 viruses are very common in birds worldwide. They represent the oldest AIV subtypes known since 1901. All H7 NA combinations were found in wild birds during surveillance in the last 40 years, where H7N7 was reported the most in more than 21 countries followed by H7N1 and H7N3 (Abdelwhab et al., 2014; Alexander, 2000; Lang et al., 1981; Webster et al., 1981). So far only low pathogenic H7Nx viruses have been found in wild ducks (Abdelwhab et al., 2014). Until the 1990s, there were only few reports on outbreaks caused by viruses of H7 subtype including the panzootic outbreaks of H7N1 from 1901 to 1930 in addition to H7N3 in 1971, 1979-1980 and H7N9 in 1988 in the USA (Banks et al., 2000; Beard and Helfer, 1972; Lupiani and Reddy, 2009). Furthermore, outbreaks of H7N1, H7N3 and H7N7 were reported in birds in UK in 1960 to 1980 (Alexander and Spackman, 1981). In Germany, H7N7 caused large outbreaks in birds in 1970s and 1980s (Banks et al., 2000).

2.4.2 Direct evolution of HPAIV H7 from LPAIV precursors

Like H5 viruses, after circulation in terrestrial poultry H7 viruses can acquire a polybasic HACS and become highly pathogenic. A comprehensive review summarizing these outbreaks in poultry in the last two decades has been published (Abdelwhab et al., 2014). The evolution of HPAIV from LP precursors in chickens has been reported during the following outbreaks (Table 1). In each of these outbreaks, HPAIV H7Nx viruses acquired an unique polybasic HACS and additional mutations in other gene segments. So far, only the genetic determinants for

virulence of the Italian H7N1 and Asian H7N9 were successfully identified (Abdelwhab el et al., 2016; Abdelwhab et al., 2016).

Table 1: Outbreaks of HPAIV H7Nx directly evolving from LP precursors from 1990s to 2019. Updated from (Abdelwhab et al., 2014)

Year	Country	Subtype	Host species	Cleavage site	Reference
1994/95	Pakistan	H7N3	Chickens	PETPKRKRKR/GLF PETPKRRKR/GLF PETPKRRNR/GLF	(Aamir et al., 2009; Abbas et al., 2010; Banks et al., 2000; Naeem and Hussain, 1995; Naeem et al., 2007)
1999	Italy	H7N1	Several poultry species	PKGSRVRR/GLF	(Capua, 2008; Capua et al., 2003; Capua and Mutinelli, 2001; Capua et al., 2002; Capua et al., 2000a; Capua et al., 2000b; Magnino et al., 2000; Zanella et al., 2001)

Year	Country	Subtype	Host species	Cleavage site	Reference
2002	Chile	H7N3	Chickens	PKT**C**SPLSRCRKTR/GLF	(Max et al., 2007; Rojas et al., 2002; Suarez et al., 2004)
2003	Netherlands	H7N7	Chickens, Humans	PKRRRR/GLF	(de Jong et al., 2009; de Wit et al., 2004; Fouchier et al., 2004; Meijer et al., 2006)
2003/2004	Pakistan	H7N3	Chickens	PETPKRRKR/GLF	(Abbas et al., 2010; Naeem et al., 2007)
2004	Canada	H7N3	Chickens, Humans	PKQAYQKRMTR/GLF	(Belser et al., 2008; Belser et al., 2009b; Pasick et al., 2005; Tweed et al., 2004)
2007	Canada	H7N3	Chickens	PKTTKPRPRR/GLF	(Berhane et al., 2009; Pasick et al., 2010)
2008	England	H7N7	Chickens	PEIPKKKKR/GLF PEIPKRKKR/GLF PEIPKKKKKKR/GLF	(Seekings et al., 2018)

Year	Country	Subtype	Host species	Cleavage site	Reference
2012	Mexico	H7N3	Chickens	PKDRKSRHRRTR/GLF	(Kapczynski et al., 2013; Maurer-Stroh et al., 2013)
2015	Germany	H7N7	Chickens	PEIPKRKRR/GLF	(Dietze et al., 2018)
2016	USA	H7N8	Turkeys, Chickens	PKKRKTR/GLF	(Killian et al., 2016)
2017	USA	H7N9	Chickens	PENPKTDRKSRHRRIR/GLF	(Belser et al., 2018; Lee et al., 2017)
2014-to date	China	H7N9	Poultry, Humans	KGKRTAR/GLF KRKRTAR/GLF	(Wang et al., 2017; Yang et al., 2017)

2.4.3 Epidemiology of H7 in Germany

2.4.3.1 H7N7 in Germany

From 2001 to 2015, independent introductions of several H7N7 viruses were reported in wild and domestic birds in Germany. In 2001, chickens, turkeys, ducks and geese in a household flock in Southern Germany were infected by H7N7, probably of wild bird origin. This infection led to culling of 145 birds. In 2003, HPAIV H7N7 caused outbreaks in poultry and humans in the Netherlands and extended to poultry in Germany and Belgium, marking this outbreak as the largest H7N7 outbreak in Europe. In Germany, 413000 chickens and ducks died or were culled (Werner et al., 2003). In 2006, LPAIV H7N7 was detected in wild birds (i.e. pochard and swans). In 2009, 16700 farmed poultry have been culled during an LPAIV infection. In 2010, LPAIV H7N7 was isolated from chickens. In May 2011, a large outbreak of LPAIV H7N7 led to the destruction of around 80000 poultry in 23 commercial and backyard premises. Infected poultry showed only mild clinical signs. In 2013, a turkey flock with 34000 birds had to be culled because of LPAIV H7N7 infection. Except for the 2003-virus, LP H7N7 viruses in other incidences were most likely introduced by wild birds (Abdelwhab et al., 2014; Probst et al., 2012; Werner et al., 2003).

2.4.3.2 Outbreak of H7N7 in Emsland, Germany 2015

In 2015, H7N7 viruses were isolated within a few weeks from chickens in two separate premises located at a distance of about 340 m. In June 2015, LP H7N7 was detected in a chicken flock, 36200 laying hens in an open rearing system, showing general sickness and decreased egg production. After the confirmation of LPAIV subtype H7N7 all birds were culled to eradicate the infection. Birds in farms in a restriction zone of 10 km tested negative for AIV. A few weeks later, birds in an adjacent farm were affected by the same virus. Both, LP and HP H7N7 viruses, were detected on this farm in July due to increased mortality overnight and reduction in egg production. Direct epidemiological link between the two farms is still missing. Hence, the first farm was an open holding with a pond in close vicinity, it is highly likely that wild aquatic birds transmitted the virus to the flock. In the second farm holding 10000 chickens in an indoor system, the virus was probably transmitted due to insufficient biosafety or the access of wild birds to the animals or their feed. All animals in this holding were also culled. Sequence analyses revealed that the LPAIV contained a monobasic CS motif PEIPKG**R**/G and the HP progeny had the polybasic motif PEIP**KRKRR**/G. Moreover, all segments of HPAIV possessed synonymous and non-synonymous mutations compared to the LPAIV (Table 2). All of them, except N92D in the NS1 protein, are of unknown biological function. Phylogenetic analyses revealed that the virus belonged to the Eurasian lineage, closely related to viruses from the Netherlands and the UK from 2015 without any reference to the zoonotic H7N9 from

China. Nevertheless the NS segment originated from a different allele than the one from outbreaks in UK.

Table 2: Mutations in LP and HPAIV H7N7 from 2015-outbreak in chickens in Germany (HACS excluded) updated from Dietze et al. (2018).

Segment	Mutation LP/HP	Location
PB2	E123K	N2-Linker, Part of the central RNA polymerase domain (Nilsson et al., 2017; Pflug et al., 2014)
PB2	I147V	N2-Linker, Part of the central RNA polymerase domain (Nilsson et al., 2017; Pflug et al., 2014)
PB2	K335R	Cap-binding domain (Pflug et al., 2014)
PB1	F254C	Palm domain in the center (Pflug et al., 2014)
PA	K185R	N-terminal Endonuclease (Pflug et al., 2014)
HA	I13S	Signal peptide (Nobusawa et al., 1991)
NP	S478F	C-terminal region (Hu et al., 2017; Ye et al., 2006)
NA	V439A	Head domain (McAuley et al., 2019)
M2	V68L	Cytoplasmic tail (Cady et al., 2009)
NS1	N92D	Binding domain for eukaryotic translation initiation factor 4GI (Zhou et al., 2010)

3 Aim of this study

The objective of this study was to increase the knowledge of virulence of AIVs subtype H7 in different bird species.

In the first publication, the virulence of two German LP and HP H7N7 viruses in birds was investigated. The HPAIV H7N7 virus evolved from an LPAIV H7N7 precursor in commercial chickens in Emsland, Germany, in 2015. The evolution from LP to HP was accompanied by changing the monobasic CS in the HA to a polybasic CS. The contribution of the HA polybasic CS to high virulence of HPAIV H7N7 in chickens, turkeys and ducks was studied. Viral RNA was extracted from the LP and HP H7N7 viruses and was transcribed to cDNA. Gene segments were amplified using PCR and cloned in a specific vector. Moreover, changing the monobasic CS of LP to a polybasic motif similar to the HPAIV H7N7 was done by site-directed mutagenesis. Three recombinant viruses were generated by transfection of cultured cells and propagation in embryonated chicken eggs. Recombinant LP, HP and LP carrying polybasic CS (LP-poly) were *in vitro* characterized to assess differences in HA cleavability, replication kinetics and cell-to-cell spread. Virulence, excretion, tissue tropism and bird-to-bird transmission of the three recombinant viruses were assessed in chickens, turkeys and ducks.

In the second publication, the susceptibility of different duck species to H7 viruses was studied. Pekin and/or Muscovy ducks were challenged intrachoanal or intramuscular with two historic H7N7 and H7N1 and one recent H7N1. Morbidity, mortality, and onset and duration of virus excretion in the oropharyngeal and cloacal swabs were described.

4 Outline of publications in this dissertation

4.1 First publication (published in Scientific Reports, 2019)

Variable impact of the hemagglutinin polybasic cleavage site on the virulence of avian influenza H7N7 virus in chickens, turkeys and ducks

David Scheibner, Reiner Ulrich, Olanrewaju I. Fatola, Annika Graaf, Marcel Gischke, Ahmed H. Salaheldin, Timm C. Harder, Jutta Veits, Thomas C. Mettenleiter and Elsayed M. Abdelwhab

In 2015, a natural pair of a low pathogenic and a highly pathogenic H7N7 virus was isolated on the same farm. In this publication, the impact of the polybasic HACS on virulence in chickens, turkeys and ducks was investigated. Using reverse genetics and site-directed mutagenesis three different viruses have been rescued: LP, HP and a LP virus carrying the HP polybasic HACS designated as LP-poly. These viruses were propagated in embryonated chicken eggs and characterized *in vitro*. Whereas all three viruses showed comparable replication kinetics, HP was more efficient in cell-to-cell spread. *In vivo* studies showed comparable results of LP and HP in chickens and turkeys, with 0% and 100% mortality, respectively. Interestingly the LP-poly virus was more virulent in chickens than in turkeys. Thus, other segments or host factors can modulate virulence in turkeys compared to chickens. Moreover, Mallard, Pekin and Muscovy ducks were inoculated with the HP virus. Only Muscovy ducks showed mild to moderate clinical signs and excreted a high amount of virus, while Pekin and Mallard ducks stayed healthy yet Muscovy ducks shed the virus via the cloacal route. NP antigen in the endothelial cells as only detected in chickens. Findings in this study suggest that virulence determinants and the underlying pathomechanism of this H7N7 virus are different in chickens and turkeys. Muscovy and Mallard ducks may serve as natural reservoir.

4.2 Second publication (published in BMC Veterinary Research, 2019)

Virulence of three European H7Nx viruses in Pekin and Muscovy ducks

David Scheibner, Claudia Blaurock, Thomas Mettenleiter, El-Sayed Abdelwhab

Compared to H5Nx viruses, the virulence of H7Nx in ducks was not adequately investigated. Given the wide spread of diverse H7 viruses in wild waterfowl in Europe including Germany, it was important to assess the virulence of historic and recent H7N1 in domestic ducks as potential reservoir. In this paper, three European HPAIV H7N1 and H7N7 have been tested in Muscovy ducks via intrachoanal inoculation. Furthermore, the effect of duck species and inoculation route was tested by infection of Pekin ducks intramuscularly or intrachoanally with an Italian H7N1 from the 1999 epidemic. While the fowl plague H7N7 virus (FPV) from 1927

killed two out of ten Muscovy ducks, H7N1-FPV 1934 was more virulent and killed eight of ten inoculated ducklings. The Italian H7N1-1999 was more virulent in Pekin than in Muscovy ducks after intrachoanal inoculation. Conversely, the virus was more virulent in Muscovy ducks than in Pekin ducks after intramuscular inoculation. Although all viruses with polybasic HACS, were HP in chickens mortality rate in ducklings varied from 20% to 80%. There is a need to determine the genetic markers for virulence determinants of H7Nx viruses in ducks.

Chapter 2

Variable impact of the hemagglutinin polybasic cleavage site on virulence and pathogenesis of avian influenza H7N7 virus in chickens, turkeys and ducks

David Scheibner, Reiner Ulrich, Olanrewaju I. Fatola, Annika Graaf, Marcel Gischke, Ahmed H. Salaheldin, Timm C. Harder, Jutta Veits, Thomas C. Mettenleiter and Elsayed M. Abdelwhab

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Variable impact of the hemagglutinin polybasic cleavage site on virulence and pathogenesis of avian influenza H7N7 virus in chickens, turkeys and ducks

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Avian influenza viruses (AIV) are classified into 16 hemagglutinin (HA; H1-H16) and 9 neuraminidase (NA; N1-N9) subtypes. All AIV are low pathogenic (LP) in birds, but subtypes H5 and H7 AIV can evolve into highly pathogenic (HP) forms. In the last two decades evolution of HPAIV H7 from LPAIV has been frequently reported. However, little is known about the pathogenesis and evolution of HP H7 from LP ancestors particularly, in non-chicken hosts. In 2015, both LP and HP H7N7 AIV were isolated from chickens in two neighbouring farms in Germany. Here, the virulence of these isogenic H7N7 LP, HP and LP virus carrying a polybasic HA cleavage site (HACS) from HP (designated LP-Poly) was studied in chickens, turkeys and different duck breeds. The LP precursor was avirulent in all birds. In contrast, all inoculated and contact chickens and turkeys died after infection with HP. HP infected Pekin and Mallard ducks remained clinically healthy, while Muscovy ducks exhibited moderate depression and excreted viruses at significantly higher amounts. The polybasic HACS increased virulence in a species-specific manner with intravenous pathogenicity indices of 3.0, 1.9 and 0.2 in chickens, turkeys and Muscovy ducks, respectively. Infection of endothelial cells was only observed in chickens. In summary, Pekin and Mallard were more resistant to HPAIV H7N7 than chickens, turkeys and Muscovy ducks. The polybasic HACS was the main determinant for virulence and endotheliotropism of HPAIV H7N7 in chickens, whereas other viral and/or host factors play an essential role in virulence and pathogenesis in turkeys and ducks.

Avian influenza A viruses (AIV) are members of the family *Orthomyxoviridae*. They are differentiated according to the antigenicity of the hemagglutinin (HA) and neuraminidase (NA) proteins into 16 HA (H1 to H16) and 9 NA (N1 to N9) subtypes[1]. HA is a surface glycoprotein which mediates virus attachment and fusion with the host cell endosomal membrane. It plays essential roles in virulence, immunogenicity and interspecies transmission (e.g. from birds to mammals)[2]. The HA is synthesized in a fusion-inactive form (HA0), which is activated through cleavage by host proteases into HA1 and HA2 subunits. The HA monobasic cleavage site (CS) of low pathogenic (LP) AIV is recognized by trypsin-like proteases in the respiratory and digestive tracts. LPAIV cause mild, if any, clinical signs, while highly pathogenic (HP) AIV carry a multibasic CS, which is cleaved by ubiquitous furin-like proteases resulting in systemic infections and high mortality[3–6]. In nature, only H5 and H7 subtypes can evolve from LP precursors to HPAIV by acquisition of mutations particularly in CS of the HA protein[4]. This transition

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mostly occurs in terrestrial poultry but can spill to wild birds[7]. Evolution of HPAIV from LPAIV is usually accompanied by mutations in other gene segments in addition to HA. These mutations are unique to each HPAIV and can modulate virulence and bird-to-bird transmission[8–12]. Therefore, it is important to study the virulence determinants of each HPAIV separately.

Recently, the prevalence of H7 viruses and the evolution of HPAIV H7 from low pathogenic precursors increased remarkably[13–17]. Turkeys are more susceptible than chickens to AIV, enabling adaptation of wild bird-origin viruses to other domestic birds and play an important role in interspecies transmission (e.g. from birds to pigs and humans and *vice versa*)[18–20]. Nevertheless, data on the virulence determinants of HPAIV in turkeys are scarce. Likewise, few studies have been conducted in domestic ducks to evaluate the pathogenicity of HPAIV H7Nx. Several HPAIV H7N3 from Chile, Canada and Mexico, H7N4 from Australia and H7N7 from the Netherlands did not cause any mortality in two-week-old Pekin ducks[21]. Similarly, Pekin and Mallard ducklings were clinically resistant to the Italian HPAIV H7N1[21,22]. Commercial young or adult Pekin ducks did not succumb to mortality with different Dutch and Australian HPAIV H7N7[23–26]. Virulence of HPAIV H7 in Muscovy ducks has not been studied before, although under natural conditions neurological disorders and high mortality during the 1999–2000 outbreaks in Italy were linked to infection by HPAIV H7N1[27]. Muscovy ducks were more sensitive to HPAIV H5N1 than Pekin ducks[28–30], probably due to differences in immune responses[29,30] which may vary according to the virus strain, age of ducks and route of virus inoculation[28,31,32]. Importantly, virulence determinants of HPAIV H5Nx in chickens differ from those in ducks[33–36].

In 2015, an HPAIV H7N7 was isolated from a commercial chicken layer flock in Germany. The virus evolved from LP H7N7, which was simultaneously isolated from the same farm. The HA of the low pathogenic virus carried a monobasic HACS (PEIPKGR/G), while the HP specified a polybasic CS (PEIPKRKRR/G)[37]. Here, the impact of the polybasic HACS on virus replication, virulence, bird-to-bird transmission and tropism was investigated in chickens, turkeys and different duck breeds.

Results

Three recombinant H7N7 viruses were successfully generated using reverse genetics. All gene segments of LPAIV A/chicken/Germany/AR915/2015(H7N7) (designated hereafter LP) and HPAIV A/chicken/Germany/AR1385/2015(H7N7) (designated hereafter HP) were successfully amplified and cloned. Moreover, to generate an LP with a polybasic CS (designated hereafter LP-Poly), the HA segment of LP was used to change the monobasic (PEIPKGR/G), to the polybasic PEIPKRKRR/G, exactly resembling the HP H7N7 CS. LP, HP and LP-Poly were successfully rescued and showed comparable titres from $10^{6.7}$ to $10^{7.0}$ plaque forming unit per ml (PFU/ml) in the allantoic fluids after propagation in specific pathogen free (SPF) embryonated chicken eggs (ECE).

The impact of a polybasic HACS on virus replication, spread and HA cleavability in cell culture. All three viruses replicated in avian and mammalian cell lines. No significant differences were observed in chicken embryo kidney (CEK) cells (Fig. 1 panel b). In Madin-Darby canine kidney cells type II (MDCKII), LP-Poly replicated to higher titres than the LP and HP viruses (Fig. 1 panel b), which, however, was not statistically significant ($P > 0.05$). The HP virus induced significantly ($P < 0.0001$) larger plaques in MDCKII than LP and LP-Poly (Fig. 1 panel c). The HA of LP was only efficiently activated in the presence of trypsin, while insertion of a polybasic cleavage site allowed cleavage of the LP HA in the absence of trypsin (Fig. 1 panel d).

Insertion of the polybasic CS increased virulence and tropism of LP H7N7 comparable to HP H7N7 after oculonasal (ON) or intravenous (IV) infection of chickens. After oculonasal (ON) inoculation with LP, chickens showed mild clinical signs (i.e. ruffled feathers and diarrhoea) for a few days and recovered quickly. All contact birds in this group survived without showing clinical signs. Conversely, 5/6 and 6/6 inoculated chickens, and 3/4 and 4/4 contact chickens died after infection with LP-Poly and HP viruses with pathogenicity indices (PI) of 1.8 and 2.2, and a mean death time (MDT) of 5 and 4.3 days, respectively (Table 1, Fig. 2 panel a). Birds showed clinical signs typical of HPAIV infection, e.g. cyanosis of comb and wattle, haemorrhages on the shanks and unfeathered parts of the body and moderate to severe depression. After intravenous (IV) injection, LP infected-birds showed transient mild clinical signs with an intravenous PI (IVPI) of 0.1 (Table 1; Fig. 2 panel b). All chickens injected with LP-Poly and HP died and the calculated IVPI was 3.0 (Table 1; Fig. 2 panel b). Using ELISA, anti-NP antibodies were detected in all surviving chickens inoculated IV and ON with LP or ON with LP-Poly (data not shown).

Virus excretion was quantified using RT-qPCR of swab samples collected at 4 days post inoculation (dpi). All chickens excreted viruses in oropharyngeal (OP) and cloacal (CL) swabs (Fig. 2 panels c–f). LP virus was detected in the oropharyngeal swabs of inoculated chickens and their cagemates at lower titres than in cloacal swabs (Fig. 2 panels c–f). The insertion of a polybasic HACS significantly ($P < 0.05$) increased LP excretion in oropharyngeal swabs to a level comparable to HP (Fig. 2 panel c).

On day 4 after ON inoculation, viral NP antigen was detected by immunohistochemistry (IHC) and histology in organs of two chickens per group (Figs 3 and 4). In parenchymal tissues, LP was detected in the intestinal tract (duodenum, jejunum and cecum) and spleen (Fig. 3 panel a). Insertion of the polybasic CS resulted in the distribution of the LP-Poly in all analysed organs at a level comparable to HP. Only LP-Poly was detected in the gizzard. The highest amount of HP and LP-Poly was detected in the brain and lungs (Fig. 3 panel a). LP was not detected in any endothelial tissue, whereas LP-Poly was present in the endothelium of all tissues resembling HP infection (Fig. 3 panel b and Fig. 4). Moreover, histopathological examination of birds inoculated with LP revealed mild inflammation in the lungs only (Fig. 3 panel d). Conversely, HP and LP-Poly induced mild to severe necrosis or necrotic inflammation in the Bursa of Fabricius, thymus, cecum, spleen (Fig. 3 panel d), pancreas and heart (Fig. 3

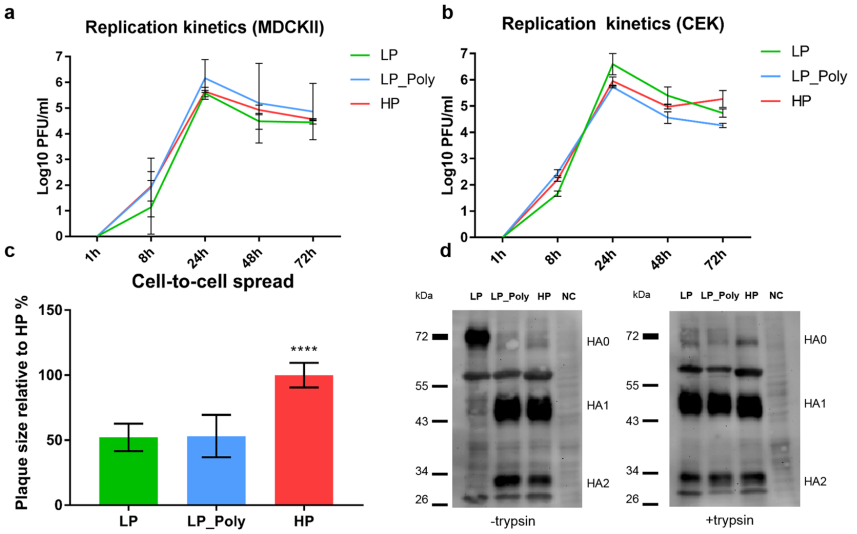

Figure 1. *In vitro* characterisation of recombinant H7N7 viruses in this study. The replication efficiency in MDCKII (**a**) and CEK (**b**) cells at an MOI of 0.001 for 1, 8, 24, 48 and 72 hours post infection is illustrated. Results are shown as the mean and standard deviations of all values of triplicates of two independent runs. Cell-to-cell spread was determined by measuring the diameter of plaques induced by the indicated viruses in MDCKII cells. The size of plaques induced by HP was set as 100%. The results are expressed as mean and standard deviation. (**c**) The cleavability of HA0 into HA1 and HA2 subunits was studied using Western Blot after the infection of MDCKII cells at an MOI of 1 PFU per cell of indicated viruses in the presence (+) or absence (−) of trypsin. The viral proteins were detected by polyclonal chicken serum against H7N1 at a ratio 1:500 after separation in a 10% polyacrylamide gel. NC refers to the mock control (non-infected cells). (**d**) The original Western Blot figure is available as a Supplementary Fig. S1.

Virus	Chickens				Turkeys				Muscovy Ducks			
	ON			IV	ON			IV	ON			IV
	Dead/ Inoculated*	Dead/ Contact*	PI (MDT; range)	IVPI	Dead/ Inoculated	Dead/ Contact	PI (MDT; range)	IVPI	Dead/ Inoculated	Dead/ Contact	PI	IVPI
LP	0/6	0/4	0.0	0.1	0/10	0/5	0.2	0.1	0/10	0/5	0.0	0.0
LP-Poly	5/6	3/4	1.8 (5; 3–9)	3.0	3/10	2/5	1.1 (5; 5)	1.9	0/10	0/5	0.0	0.2
HP	6/6	4/4	2.2 (4.3; 3–5)	3.0	10/10	5/5	2.2 (5; 5)	2.8	0/10	0/5	0.1	0.5

Table 1. Clinical scoring after oculonasal (ON) or intravenous (IV) infection of chickens, turkeys and Muscovy ducks *Number of dead birds to the total number of inoculated or contact birds. ON = oculonasal, IV = intravenous, PI = pathogenicity index, IVPI = intravenous pathogenicity index, MDT = mean death time for primarily inoculated birds, "range" refers to the first and last day when birds died. Birds were observed daily and the severity of clinical signs were given scores from 0 (no signs) to 3 (dead). PI and IVPI values range from 0 (avirulent) to 3 (high virulent) and were calculated by dividing the sum of the arithmetic mean values of daily scores of inoculated birds by 10 (the number of observation days) according to the OIE protocol[56].

panel c). Moreover, only HP caused lesions in the jejunum, proventriculus, duodenum, liver, brain and trachea (Fig. 3 panels c and d).

In turkeys, the LP with polybasic HACS exhibited lower virulence than in chickens and was not detected in the endothelium of any organ. After ON inoculation of turkeys with LP transient mild clinical signs (i.e. ruffled feather and slight depression) were observed in inoculated and contact animals which quickly recovered. None of the turkeys died. All turkeys inoculated ON with HP showed severe depression and central nervous signs (i.e. opisthotonus, torticollis, paralysis) at 4 dpi. They were humanely killed and scored as dead at 5 dpi resulting in a pathogenicity index (PI) of 2.2. All contact turkeys died within 7 days. Interestingly, only 3/10 inoculated and 2/5 contact turkeys died after ON inoculation with LP-Poly. The PI value for this virus was 1.1 which was significantly less than in chickens ($P < 0.05$) (Fig. 2 panel a). As was observed in chickens, the

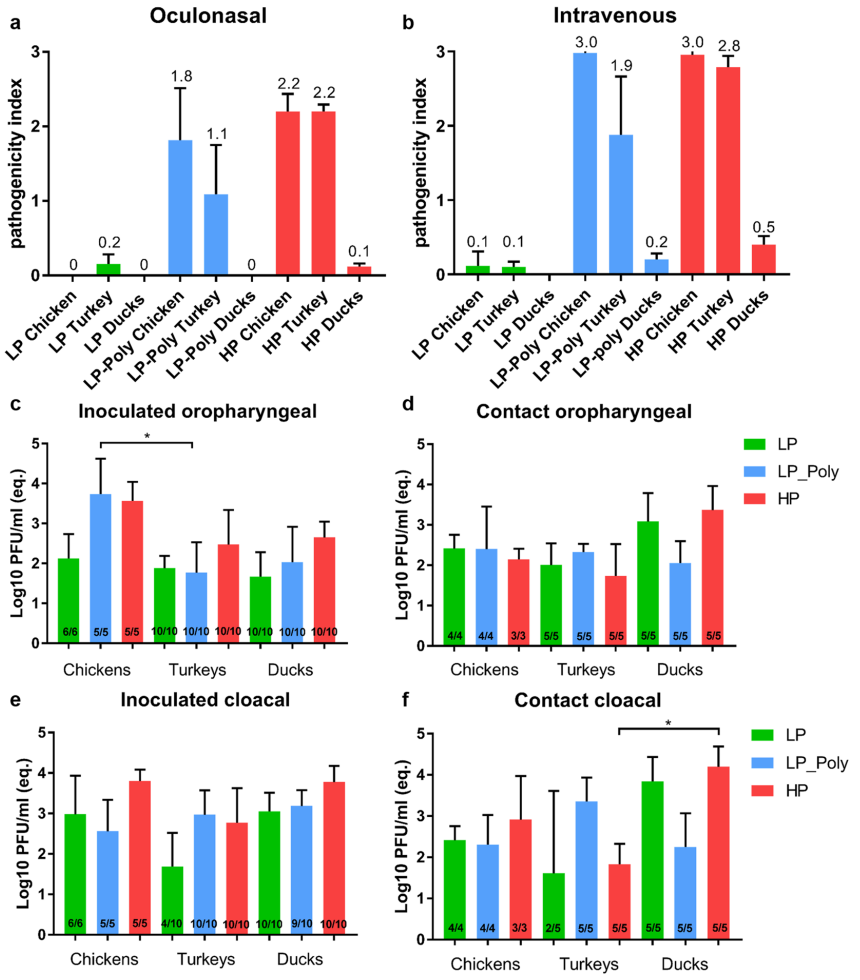

Figure 2. Clinical scoring and virus excretion in experimentally infected chickens, turkeys and Muscovy ducks. Clinical scoring after oculonasal (**a**) or intravenous (**b**) infection was calculated by dividing the sum of the arithmetic mean values of daily scores by 10 (the number of observation days). The PI and IVPI values for each virus ranged from 0 (avirulent) to 3 (highly virulent) and the results are expressed as mean and standard deviation. Virus excretion at 4 days post inoculation in oropharyngeal (**c**,**d**) and cloacal (**e**,**f**) swabs of inoculated (c and e) and contact (d and f) birds was determined by RT-qPCR against standard curves using ten-fold dilutions of HPAIV H7N7 and the mean and standard deviations were expressed as equivalent plaque forming unit pro ml (eq. PFU/mL). Asterisks indicate significant differences at $p \leq 0.05$ (*) or 0.01 (**) or 0.0001 (****).

IVPI of LP and HP were 0.1 and 2.8, respectively, whereas LP-Poly killed only 5 out of 9 birds with an IVPI of 1.9 which was remarkably less than in chickens (Table 1; Fig. 2 panels a and b). All surviving turkeys seroconverted as demonstrated by the detection of anti-NP antibodies by ELISA (data not shown).

Viruses were detected at similar levels in oropharyngeal swabs from inoculated and contact animals (Fig. 2 panels c and d). Like HP inoculated turkeys, LP-Poly inoculated birds excreted higher amounts of viruses via the cloacal route than LP inoculated birds, whereas in-contact turkeys excreted LP-Poly at higher levels than either LP or HP. Compared to chickens, inoculated turkeys oropharyngeal excretion of LP-Poly and HP was about 10- to 100- times decreased, whereas LP and HP were found at about 10 times decreased amounts in the cloacal swabs. LP-Poly and HP were detected at comparable amounts in both cloacal and oropharyngeal swabs, but contact turkeys excreted LP-Poly in cloacal swabs at higher levels than LP and HP (Fig. 2 panels c–f). LP was detected in the cloacal swabs of 4/10 and 2/5 inoculated and contact turkeys, respectively, and in oropharyngeal swabs of all turkeys. All turkeys excreted LP-Poly and HP in cloacal and oropharyngeal swabs.

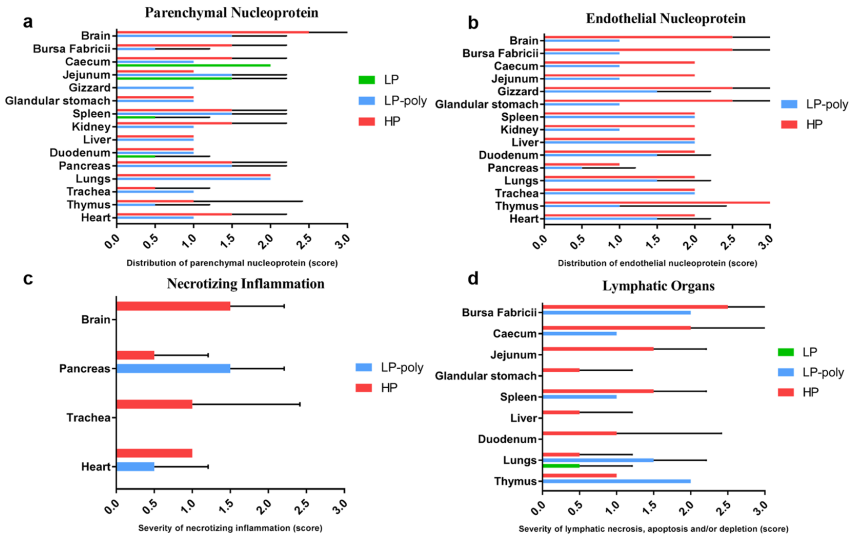

Figure 3. Distribution of influenza A antigen in different organs of inoculated chickens. Distribution of NP antigen in the parenchyma (**a**) and endothelium (**b**) of different organs of two inoculated chickens at 4 days post inoculation was detected by immunohistochemistry. The intensity and distribution of NP signals are scaled 0 (no signal), 1 (focal to oligofocal), 2 (multifocal) or 3 (confluent to diffuse). The severity of lymphatic necrosis, apoptosis and/or depletion (**c**) and necrotizing inflammation (**d**) was assessed by histopathological examination on a scale 0 (negative), 1 (low), 2 (moderate) or 3 (high). Values are shown as median and standard deviation scores of two chickens.

Using IHC, a focal to oligofocal distribution of the NP antigen of LP was observed only in the cecal epithelium and parenchyma of pancreas. The distribution of LP-Poly in the parenchyma of different organs was similar to HP, although at slightly lower levels (Figs 5 and 6). The highest amount of LP-Poly was detected in the brain, cecum and pancreas, while HP was concentrated in the brain, cecum, kidneys and heart. Interestingly, like in chickens, only LP-Poly was detected in the gizzard parenchyma in addition to the jejunum (Fig. 5 panel a). Remarkably, and in stark contrast to chickens, none of the viruses were detected in the endothelium of any organ of examined turkeys (Fig. 6). No pathological changes were observed in turkeys inoculated with LP at 4dpi. LP-Poly and HP caused comparable lymphatic necrosis, apoptosis and/or depletion in the bursa, cecum and thymus and comparable mild to severe necrotic encephalitis, pancreatitis and myocarditis (Fig. 5 panels b and c). Nevertheless, only HP caused low to severe necrosis in the spleen and lungs (Fig. 5 panel c).

Muscovy ducks were more susceptible than Pekin and Mallard ducks and excreted virus at significantly higher levels. Virulence of HP was assessed in Pekin, Mallard and Muscovy ducks after ON and/or IV infection. After ON inoculation of Muscovy ducks with LP, LP-Poly and HP, five sentinel Muscovy ducks were added to assess bird-to-bird transmission. Ducks that were oculonasally inoculated with LP and LP-Poly did not show any clinical signs or mortality. After ON challenge with HP, only Muscovy ducks exhibited a transient mild depression with a PI 0.1, while Pekin and Mallard ducks remained clinically healthy. None of the contact Muscovy ducks showed clinical signs. After IV injection, only Muscovy ducks exhibited mild to moderate depression, reluctance to move and steady gait after infection with HP, and to a lesser extent with LP-Poly with IVPI values of 0.5 and 0.2, respectively (Table 1).

HP was detected in cloacal swabs at higher levels than in the oropharyngeal swabs in all duck breeds (Fig. 7 Panel a). Pekin ducks excreted the lowest amount of virus and presented the lowest number of shedders. Eight Mallard ducks were positive in cloacal swabs but only one duck was positive in oropharyngeal swabs. Mallard ducks presented higher amounts of virus in cloacal than Pekin ducks. All Muscovy ducks excreted significantly higher amounts of HP oropharynx compared to Mallards and Pekin ducks (Fig. 7 Panel a). Moreover, significantly higher virus titres were found in cloacal swab samples of the Muscovy ducks compared to Pekin ducks. After the ON inoculation with HP, LP, and LP-Poly, viral RNA was detected in oropharyngeal and cloacal swabs from all inoculated Muscovy ducks except one LP-Poly inoculated duck (Fig. 7 Panel b). The average amount in the cloacal swabs was higher than in the oropharyngeal swabs of all viruses (Fig. 7 Panel b). All contact Muscovy ducks were positive in oropharyngeal and cloacal swabs but HP and LP viruses were present in higher amounts in oropharyngeal and cloacal swabs of contact birds compared to LP-Poly (Fig. 7 Panel c). At the termination of the experiment all ON and IV inoculated Pekin and Mallard ducks had anti-NP antibodies as detected by ELISA. Likewise, all Muscovy ducks infected with LP, LP-Poly and HP as well as contact animals seroconverted (Fig. 7 Panel d).

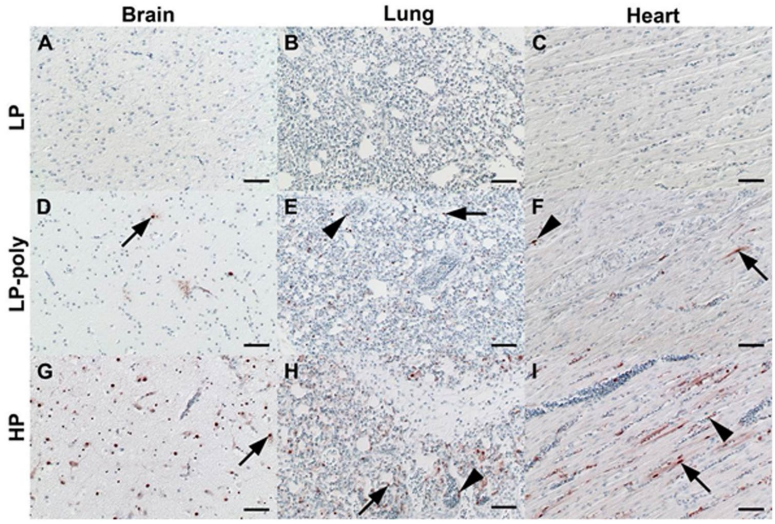

Figure 4. Distribution of influenza A NP antigen in different organs in chickens. Distribution of influenza A NP antigen in different organs in chickens at 4 days post inoculation with LP (**a**–**c**), LP-Poly (**d**–**f**) or HP (**g**–**i**) displaying variable level of organ tropism ranging from a minimum of none to a maximum of coalescing nucleoprotein within brain (**a**,**d**,**g**), lung (**b**,**e**,**h**) and heart (**c**,**f**,**i**). Arrows point to parenchymal cells with influenza A virus nucleoprotein-positive nuclei (neuroglia cells, pneumocytes or cardiomyocytes). Arrowheads point to endothelial cells with influenza A virus nucleoprotein-positive nuclei. Immunohistochemistry; avidin-biotin-peroxidase complex method; polyclonal rabbit anti-influenza A FPV/Rostock/34-virus-nucleoprotein antiserum[61]; 3-amino-9-ethyl-carbazol (red-brown); hematoxylin counterstain (blue); Nomarski contrast; Bars = 50 µm.

NP detection by IHC was done only for Muscovy ducks. Antigen was not detected in endothelial cells in any Muscovy duck and neither microscopic lesions nor antigen were found in the gizzard or brain. After inoculation with LP, a focal distribution of NP was detected in the epithelium of cecum and trachea (score 1). Histopathological changes were observed in at least one bird including lymphohistiocytic infiltration in the trachea and proventriculus with mild lymphoid depletion in the bursa of Fabricius and thymus. Inoculation of Muscovy ducks with LP-Poly resulted in focal distribution of NP antigens in the parenchyma of thymus, lung, jejunum, cecum and/or bursa of Fabricius of at least one bird. Furthermore, trachea had moderate multifocal degeneration of the epithelium with loss of cilia with lymphocytic infiltration of the lamina propria. In the lungs, hyperplasia of bronchial associated lymphocytes (BALT) and bronchitis with epithelial degeneration and neutrophils infiltration were seen. The pancreas, liver and kidneys contained a mild focal lymphohistiocytic infiltrate and the duodenum had focal lymphocytic aggregation in the gut-associated lymphocytes (GALT). Ducks inoculated with HP had antigen prominently in the thymus and trachea. The trachea exhibited moderate multifocal epithelial degeneration with loss of cilia and lymphocytic infiltration. In the lungs, mild interstitial oedema and congestion with mild lymphatic depletion of BALT was observed. Moreover, mild, acute, oligofocal lymphohistiocytic infiltration in the pancreas and mild lymphoid depletion in the proventriculus were found (data not shown).

Discussion

In the last few years evidence for direct evolution of HP H7 from LP precursors has been reported[14–16,38]. H7N7 viruses have been very prominent (excluding the current panzootic H5Nx Goose/Guangdong outbreak) during the last decades in Europe causing diseases in poultry and humans[39]. However, little is known about the mechanism underlying the shift of virulence of H7 viruses particularly in turkeys and ducks. In 2015, both HP and LP H7N7 viruses were isolated simultaneously from two neighbouring chicken layer flocks in Germany representing a rare natural isogenic precursor-progeny LPAIV/HPAIV pair. Compared to the LP precursor, the resulting HP had acquired a polybasic HACS but specified, in addition, 10 amino acids alterations namely E123K, I147V, K355R in PB2, F254C in PB1, K185R in PA, I13S in HA, S478F in NP, V439A in NA, V68L in M2 and N92D in NS1 37. In this study, the virulence of the pair of LP and HP viruses, and the impact of the polybasic HACS on virulence and transmission in chickens, turkeys and ducks were investigated.

In previous studies, we have shown that three German LP H7N7 viruses from 2003, 2011 and 2013 required only a specific polybasic HACS to exhibit full virulence equivalent to a genuine HPAIV[12]. Conversely, the insertion of different polybasic HACS motifs in an LP H7N7 isolated in 2001 from a small backyard turkey flock in Germany[12] and in an Italian LP H7N1 virus of 1999[8] did not result in full virulence of these viruses. Similarly, several H5N1 and H5N2 viruses with authentic or synthetic polybasic HACS were avirulent in chickens[9,40]. Other

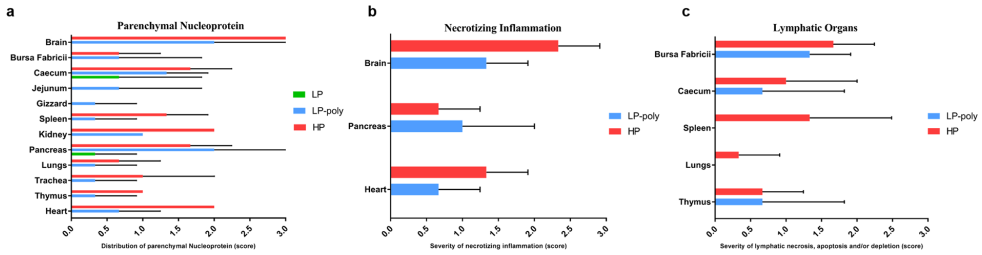

Figure 5. Distribution of influenza A antigen in different organs of inoculated turkeys. Distribution of NP antigen in the parenchyma (**a**) of different organs of three inoculated turkeys at 4 days post inoculation was detected by immunohistochemistry. NP was not detected in the endothelium of any organ of any of the three examined turkeys. The intensity and distribution of NP signals are scaled 0 (no signal), 1 (focal to oligofocal), 2 (multifocal) or 3 (confluent to diffuse). The severity of necrotizing inflammation (**b**) and lymphatic necrosis, apoptosis and/or depletion (**c**) was assessed by histopathological examination on a scale 0 (negative), 1 (low), 2 (moderate) or 3 (high). Values are shown as median and standard deviation scores of three turkeys.

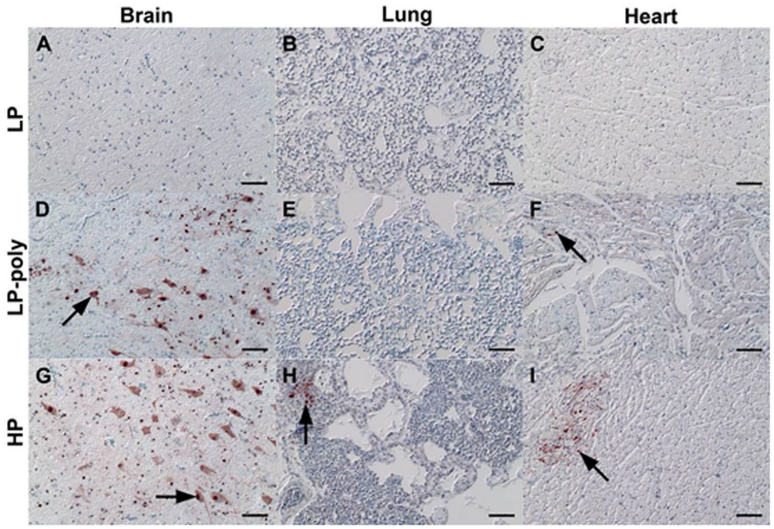

Figure 6. Distribution of influenza A NP antigen in different organs in turkeys. Distribution of influenza A NP antigen in different organs in turkeys at 4 days post inoculation with LP (**a**–**c**), LP-Poly (**d**–**f**) or HP (**g**–**i**) displaying variable level of organ tropism ranging from a minimum of none to a maximum of coalescing nucleoprotein within brain (**a**,**d**,**g**), lung (**b**,**e**,**h**) and heart (**c**,**f**,**i**). Arrows point to parenchymal cells with influenza A virus nucleoprotein-positive nuclei (neuroglia cells, macrophages/pneumocytes or cardiomyocytes). Immunohistochemistry; avidin-biotin-peroxidase complex method; polyclonal rabbit anti-influenza A FPV/Rostock/34-virus-nucleoprotein antiserum 61; 3-amino-9-ethyl-carbazol (red-brown); hematoxylin counterstain (blue); Nomarski contrast; Bars = 50 µm.

mutations in the HA1, HA2, NA or other viral genes were required for expression of high virulence in inoculated and/or sentinel chickens[9,41,42]. We show here that the insertion of the polybasic HACS increased virulence of the LP in chickens to a level comparable with the HP as indicated by IVPI and PI values. While LP was limited to the respiratory and intestinal tracts, insertion of the polybasic HACS resulted in efficient proteolytic activation *in vitro* and systemic distribution resembling the HP infection particularly in the lungs, brains and endothelial cells. These results indicate that the polybasic HACS is the main virulence determinant of this HP H7N7 in chickens, and that other mutations play at most a minimal role in virulence and tropism.

In turkeys, the virulence of LP and HP was comparable to or slightly higher than in chickens. Turkeys are more vulnerable to LP and HP AIV[43,44] but it is largely unknown whether virulence markers in both "galliform" species are different. Although it is widely accepted that chickens and turkeys are similar in their high susceptibility to AIV, these hosts are not identical. For example, chickens exhibit high susceptibility to Newcastle disease

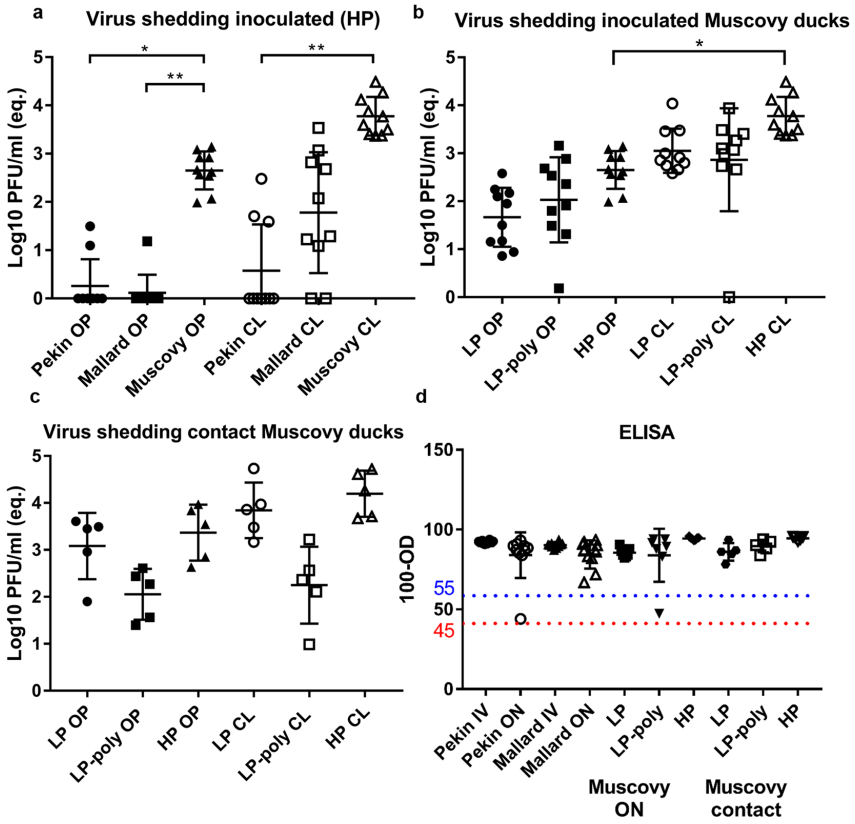

Figure 7. Virus excretion and seroconversion in inoculated and/or contact Pekin, Mallard and Muscovy ducks. Shown are the results of virus excretion at 4 days post inoculation in oropharyngeal (OP) and cloacal (CL) swabs using RT-qPCR expressed by equivalent Log10 PFU/mL in oculonasal (ON) inoculated Pekin, Mallard and Muscovy ducks with HPAIV H7N7. (**a**) Excretion of LP, LP-Poly or HP in inoculated (**b**) and contact (**c**) Muscovy ducks were also determined by RT-qPCR. All values are shown as mean and standard deviation of positive samples. Antibody titres at the end of the experiment in ducks inoculated ON or intravenously (IV) were measured by an NP antibody inhibition ELISA at the end of the experiments. Results are shown as 100- optical density (OD) reading. Lower and upper dashed lines indicate the 45–55% cut-off ratio where samples lower than 45% are negative, from 45 to 55% are questionable and over 55% are positive. (**d**) Statistical significance shown in asterisk indicate P values ≤ 0.05 (*), ≤ 0.01 (**), ≤ 0.001 (***) or ≤ 0.0001 (****).

virus, infectious bursal disease virus and infectious laryngeotracheitis virus with severe morbidity and mortality. Nevertheless, these viruses are apathogenic or significantly less virulent in turkeys[45–47]. In the current study, we demonstrate that insertion of a polybasic HACS increased virulence, excretion, tropism and transmission of the LP virus in turkeys. However, this LP-Poly was significantly less virulent than in chickens as demonstrated by lower IVPI and PI values. Intriguingly, only HP-and LP-Poly-inoculated turkeys exhibited neurological signs and no viruses were detected in the endothelial cells of any organ in turkeys suggesting a different pathogenesis of HPAIV in turkeys compared to chickens. This could be the result of virus spread to the brain via the nervous system in turkeys and via the blood stream in chickens. These results are congruent with recent findings of Pantin-Jackwood, *et al.*[43] who reported neurological signs in turkeys but not in chickens or Mallards after the inoculation with HPAIV H7N8 from the 2016-outbreak in the USA. Therefore, the contribution of host factors to the pathogenesis of H7 viruses in turkeys merits further investigations.

Here, we demonstrated that Mallard and Pekin ducks excreted considerable amounts of HP without showing clinical signs supporting the role of anatid ducks as reservoir for HPAIV[21,28]. It is important to mention that all gene segments of the precursor LP H7N7 were closely related to AIV of wild duck origin indicating a high level of adaptation to ducks[37] (unpublished data). Interestingly, LP also replicated in and was excreted from Muscovy ducks at the same high level as HP. A higher susceptibility of Muscovy ducks compared to Pekin or Mallard ducks to the 2013 Asian LPAIV H7N9 has been described[48]. Generally, independent of the duck species, excretion

through the cloaca was higher than via the oral cavity, which is in accordance with the preferential replication of some AIV in the digestive tract of ducks[49,50]. The IVPI of HP H7N7 in Muscovy ducks was 0.5 indicating moderate virulence. LP-Poly exhibited a slight increase in virulence but was still slightly less virulent than field isolated HPAIV H7N7. This demonstrates that acquisition of the polybasic HACS is the crucial mutation in the transition of LP to HP AIV. However, it also shows that other mutations play a role in determining, in a species-specific manner, high pathogenicity. Contribution of mutations in PA or PB1, in addition to HA, to the high virulence and/or efficient transmission of HPAIV H5N1 in ducks was reported[33–36,51,52]. Resistance of ducks, particularly Mallards, to HPAIV H5N1 was linked to several host immune factors including interferon-induced transmembrane proteins (IFITM) and retinoic acid-inducible gene I (RIG-I) which are absent in chickens[53,54]. Therefore, it is interesting to study the variation of these host factors in different duck species, particularly in Muscovy ducks.

In summary, the recent German HP H7N7 exhibited comparably high virulence in chickens and turkeys, while Pekin and Mallard ducks did not show overt clinical signs. Infected Muscovy ducks developed moderate illness. Tropism of H7N7 viruses to the endothelium was observed in chickens but not in turkeys or ducks. Insertion of a polybasic cleavage site into the HA of the precursor LPAIV H7N7 resulted in increased virulence to different levels in a species-specific manner. In chickens, virulence of LP carrying a polybasic HACS was comparable to the field isolated HPAIV H7N7 supporting the relevance of acquisition of a polybasic cleavage in the transition from LP to HP AIV in chickens. However, in turkeys (and to lesser extent in Muscovy ducks) the virulence increased but significantly less than in chickens suggesting the contribution of other viral or host factors in virulence and pathogenesis of H7N7 in these two species.

Materials and Methods

Viruses and cells. LP and HP H7N7 isolated from two commercial layer flocks in Emsland, Germany[37] were obtained from the National Reference Laboratory for Avian Influenza, Friedrich-Loeffler-Institut (FLI), Greifswald Insel-Riems, Germany. MDCKII used for virus titration and in combination with HEK293T cells for virus rescue were obtained from the Cell Culture Collection in Veterinary Medicine of the FLI. Primary CEK cells were prepared from 18-day-old embryonated chicken eggs as previously done[55] with few modification. Briefly, embryos were decapitated and the kidneys were obtained using sterile scissors and forceps. After two times incubation with warm trypsin at 37 °C for 20 to 25 minutes, the cells were isolated through a sterile gauze. After centrifugation at 1200 rpm for 5 minutes, the cell pellet was resuspended in minimum essential medium (MEM) containing 10% foetal calf serum (FCS), antibiotics (penicillin and streptomycin) and anti-mycotics (amphotericin).

Virus propagation. All viruses were propagated in the allantoic sac of SPF ECE purchased from VALO BioMedia GmbH (Osterholz-Scharmbeck, Germany) according to the standard protocol[56]. The allantoic fluid was collected and the hemagglutination activity was measured using 1% chicken erythrocytes[56]. Aliquots of virus stocks were kept at −70 °C until use. Viruses with polybasic HACS were propagated and handled in biosafety level 3 (BSL3) laboratory at the FLI.

SPF ECE were inoculated with the supernatant of transfected cells and candled daily. Dead embryos were kept in the refrigerator for at least one day. Subsequently the allantoic fluids were harvested and checked for bacterial contamination by culturing on Columbia sheep blood agar plates (VWR International, Germany).

Generation of recombinant viruses. Recombinant viruses were generated by reverse genetics as previously described[57,58]. Briefly, RNA was extracted from LP and HP AIV using the Qiagen RNeasy Kit (Qiagen, Germany) and transcribed to cDNA using the Omniscript Reverse Transcription Kit (Qiagen). All gene segments were amplified, cloned into the plasmid pHWS*ccdB*[57] and transformed into *E. coli* TOP10™ (Invitrogen, USA), XL1-Blue™ or SURE2™ (Stratagene, Netherlands). Plasmids containing LP or HP gene segments were extracted using Plasmid Mini or Maxi kits (Qiagen). Moreover, the insertion of polybasic CS resembling the HP was introduced in the HA gene of LP by QuikChange II XL Site-Directed Mutagenesis Kit (Invitrogen). Primers used for mutagenesis are available upon request. All recombinant viruses were rescued in HEK293T and MDCKII co-culture using Lipofectamine® 2000 and OptiMEM (Fischer Scientific, Germany)[57]. Three viruses were successfully generated: LP, HP and LP containing the polybasic CS (designated LP-Poly). Absence of undesired fortuitous mutations in all gene segments of stock viruses was confirmed by Sanger sequencing and comparison to the wild-type viruses using Geneious software (Biomatters, New Zealand).

Virus titration. Viruses were titrated by plaque assay. Confluent MDCKII cells in 12-well plates were infected with ten-fold serial dilutions of specified viruses for an hour at 37 °C/5% CO_2. Cells were overlaid with semisolid Bacto™ Agar (BD, France) containing minimal essential medium (MEM), $NaHCO_3$, non-essential amino acids, NA-pyruvate and 4% bovine serum albumin (BSA) (MP Biomedicals, USA). For propagation of LPAIV, 2 μg/mL of N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma, Germany) was added. All plates were incubated for three days at 37 °C. Cells were fixed by 10% formaldehyde containing 0.1% crystal violet. Plaques were counted and viral titres were expressed as plaque forming units per ml (PFU/ml). For measurement of plaque size Nikon NIS-Elements software was used. Up to 100 plaques were measured for each recombinant virus and the results are shown as the mean and standard deviation relative to HP virus.

Replication kinetics. MDCKII and primary CEK cells were infected at a multiplicity of infection (MOI) of 0.001 for one hour at 37 °C/5% CO_2. The inoculum was removed and the cells were incubated for two minutes with citric acid buffer pH = 3.0 to inactivate the extracellular virions. Cells were washed twice with PBS, covered by MEM containing 0.2% BSA (Sigma) and incubated at 37 °C/5% CO_2 for 1, 8, 24, 48 and 72 hours post infection (hpi). The cells and supernatant were harvested and stored at −70 °C. The assay was run in triplicates in two

independent rounds for each virus in each type of cells. Virus titres were determined using plaque assay and expressed as mean titres with standard deviation.

Western blot. The HA cleavage-activation of the three viruses was assessed after infection of MDCKII cells with an MOI of 1 using Western Blot[59]. Briefly, cells were harvested after 24 hpi. After washing with PBS and two centrifugation rounds at 6000 rpm/10 minutes, the cell pellet was suspended in Laemmli buffer (Serva, Germany) and PBS (1:1) and incubated at 95 °C for 10 minutes. Finally the cells were centrifuged at 14000 rpm for 5 minutes. The viral proteins were separated from cell lysate by sodium dodecyl sulphate 10% polyacrylamide gels and then electrotransferred to nitrocellulose membranes using a TransBlot cell (BioRad, USA). The H7 AIV proteins were detected using a polyclonal chicken anti-H7N1 serum at a concentration of 1:500 and peroxidase conjugated rabbit anti-chicken IGY^{++} antibodies (Dianova, Germany) at a concentration of 1:20000 in TBS-T. Immunodetection was achieved by chemiluminescence using Clarity™ Western ECL Substrate (BioRad, USA) and images were captured using a Bio-Rad VersaDoc Imaging System and Quantity One software.

Animal experiments. Animal experiments were performed after approval by the authorized ethics committee of the State Office of Agriculture, Food Safety, and Fishery in Mecklenburg – Western Pomerania. The commissioner for animal welfare at the FLI representing the Institutional Animal Care and Use Committee (IACUC) also approved the animal experiments, which were performed in accordance with the German Regulations for Animal Welfare.

Chickens. SPF ECE from white leghorn chickens were purchased from VALO BioMedia GmbH and incubated for 21 days at the hatchery facilities of the FLI. Six week-old chickens were divided in separate groups with ten animals each. Food and water were added *ad-libitum*. For the ON infection 6 chickens were inoculated with 0.2 ml of a virus dilution in PBS containing 10^5 PFU/chicken via the oculonasal route. At 1 dpi, four sentinel chickens were added to assess virus transmission. The IVPI was measured by IV injection of ten chickens according to the OIE recommendations[56]. All birds were observed daily for ten days and clinical scoring was done on a scale of 0, 1, 2 and 3[8]. Briefly, healthy birds were given score (0), sick birds showing one clinical sign (e.g. diarrhea, nervous manifestations, respiratory disorders) were given score (1), severely sick birds showed more than one clinical sign were given score (2) and dead birds were given score (3). The pathogenicity index (PI) was calculated by dividing the sum of the arithmetic mean values of daily scores by 10 (the number of observation days). The PI for each virus ranged from 0 (avirulent) to 3 (highly virulent).

Turkeys. Commercially available one-day-old white-breasted turkeys were purchased and kept at the FLI animal facilities. Six week-old turkeys were inoculated with the different viruses via ON or IV routes. Ten birds were inoculated ON and 1dpi five sentinel turkeys were added to each group. Moreover, eight week-old turkeys were injected IV to determine the IVPI after OIE regulations and clinically scored as mentioned above.

Ducks. One-day old Pekin, Mallard and Muscovy ducks were purchased from a local commercial source. At the FLI, faecal samples were collected from all ducks and examined to exclude, among others, infection with AIV and Salmonella spp. Each experimental room contained a swimming pool filled daily with fresh water. Two to three weeks-old, ten (Pekin and Mallard) or 15 (Muscovy) ducks were allocated to separate groups. At day 0, ten birds were inoculated with 0.2 mL containing 10^5 PFU via the ON route. For assessing transmissibility of LP, LP-Poly and HP in Muscovy ducks, on day 1 after inoculation of ten Muscovy ducks, five sentinel Muscovy ducks were added. All animals were observed for up to 14 days and clinically scored as mentioned above. To determine the IVPI of indicated viruses, ten 4-to-5-week-old ducks from each breed were injected in the wing vein as previously described[56].

Virus excretion. Oropharyngeal (OP) and cloacal (CL) swabs were collected from ON inoculated birds and their contact peers at 4 dpi and stored at −70 °C until use. The quantity of virus excretion in swab samples was determined using generic real-time reverse-transcription polymerase chain reaction (RT-qPCR)[60]. The RNA was extracted from swab media using NucleoSpin® 8/96 Virus PCR Clean-up Core Kit (Macherey & Nagel GmbH, Germany) in Tecan Freedom Evo System (Tecan, Switzerland). Standard curves using HPAIV H7N7 were run in each RT-qPCR round. The relative amount of excreted virus was quantified by plotting the Ct-values in the standard curves and the results are expressed as the average and standard deviation equivalent $\log_{10}$ PFU/ml.

Serological examination. Blood was collected at the end of the experiments from all surviving birds after euthanization using isoflurane® (CP-Pharma, Germany). Sera were tested for anti-AIV nucleoprotein (NP) antibodies using enzyme-linked immunosorbent assay (ELISA) by ID screen Influenza A Antibody Competition Multispecies kit (IDvet, France). Plates were read in a Tecan® ELISA reader. The cut-off point according to the manufacture guideline was 55%, samples between 45 and 55% were considered questionable and samples lower than 45% were considered negative.

Histopathology and immunohistochemistry. To determine the distribution of the different viruses in organs from chickens, turkeys and Muscovy ducks inoculated ON with LP, HP and LP-Poly, samples from trachea, lungs, pancreas, heart, liver, spleen, kidneys, proventriculus, gizzard, duodenum, jejunum, cecum, bursa of Fabricius, thymus and brain from primarily infected chickens (n = 2), turkeys (n = 3) and ducks (n = 3) at 4 dpi were fixed in 10% formalin and embedded in paraffin using standard methods. 5 μm sections were stained by hematoxylin and eosin, and screened for histopathological changes, whereas other sections were subjected to immunohistochemical examination using primary rabbit anti-NP (1:750) antibodies and secondary biotinylated

goat anti-rabbit IgG1 (1:200) (Vector, USA)[61]. The level of nucleoprotein antigen was estimated on a 0 to 3 scale: 0 = negative; 1 = focal to oligofocal, 2 = multifocal or 3 = confluent to diffuse and the severity of necrotizing inflammation on a scale of 0 to 3: 0 = no obvious change; 1 = mild, 2 = moderate or 3 = severe as done before[62].

Statistics. Statistical differences were analysed using a Kruskal-Wallis with post hoc Tukey tests. Results were considered statistically significant at p value ≤ 0.05. All analysis was done by GraphPad Prism software 7.04 (La Jolla California USA, www.graphpad.com).

References

1. Fouchier, R. A. *et al.* Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J Virol* **79**, 2814–2822, https://doi.org/10.1128/JVI.79.5.2814-2822.2005 (2005).
2. Bottcher-Friebertshauser, E., Garten, W., Matrosovich, M. & Klenk, H. D. The hemagglutinin: a determinant of pathogenicity. *Curr Top Microbiol Immunol* **385**, 3–34, https://doi.org/10.1007/82_2014_384 (2014).
3. Gibson, C. A., Daniels, R. S., Oxford, J. S. & McCauley, J. W. Sequence analysis of the equine H7 influenza virus haemagglutinin gene. *Virus Res* **22**, 93–106 (1992).
4. Steinhauer, D. A. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology* **258**, 1–20, https://doi.org/10.1006/viro.1999.9716 (1999).
5. Bosch, F. X., Garten, W., Klenk, H. D. & Rott, R. Proteolytic Cleavage of Influenza-Virus Hemagglutinins - Primary Structure of the Connecting Peptide between Ha1 and Ha2 Determines Proteolytic Cleavability and Pathogenicity of Avian Influenza-Viruses. *Virology* **113**, 725–735, https://doi.org/10.1016/0042-6822(81)90201-4 (1981).
6. Alexander, D. J. An overview of the epidemiology of avian influenza. *Vaccine* **25**, 5637–5644, https://doi.org/10.1016/j.vaccine.2006.10.051 (2007).
7. Swayne, D. E. Understanding the complex pathobiology of high pathogenicity avian influenza viruses in birds. *Avian Dis* **51**, 242–249, https://doi.org/10.1637/7763-110706-REGR.1 (2007).
8. Abdelwhab el, S. M. *et al.* A Unique Multibasic Proteolytic Cleavage Site and Three Mutations in the HA2 Domain Confer High Virulence of H7N1 Avian Influenza Virus in Chickens. *J Virol* **90**, 400–411, https://doi.org/10.1128/JVI.02082-15 (2016).
9. Stech, O. *et al.* The Neuraminidase Stalk Deletion Serves as Major Virulence Determinant of H5N1 Highly Pathogenic Avian Influenza Viruses in Chicken. *Sci Rep* **5**, 13493, https://doi.org/10.1038/srep13493 (2015).
10. Abdelwhab el, S. M., Veits, J. & Mettenleiter, T. C. Genetic changes that accompanied shifts of low pathogenic avian influenza viruses toward higher pathogenicity in poultry. *Virulence* **4**, 441–452, https://doi.org/10.4161/viru.25710 (2013).
11. Diederich, S. *et al.* Hemagglutinin-Neuraminidase Balance Influences the Virulence Phenotype of a Recombinant H5N3 Influenza A Virus Possessing a Polybasic HA0 Cleavage Site. *J Virol* **89**, 10724–10734, https://doi.org/10.1128/JVI.01238-15 (2015).
12. Abdelwhab, E. M. *et al.* Composition of the Hemagglutinin Polybasic Proteolytic Cleavage Motif Mediates Variable Virulence of H7N7 Avian Influenza Viruses. *Sci Rep* **6**, 39505, https://doi.org/10.1038/srep39505 (2016).
13. Monne, I. *et al.* Emergence of a highly pathogenic avian influenza virus from a low-pathogenic progenitor. *J Virol* **88**, 4375–4388, https://doi.org/10.1128/JVI.03181-13 (2014).
14. Seekings, A. H. *et al.* Direct evidence of H7N7 avian influenza virus mutation from low to high virulence on a single poultry premises during an outbreak in free range chickens in the UK, 2008. *Infect Genet Evol* **64**, 13–31, https://doi.org/10.1016/j.meegid.2018.06.005 (2018).
15. Killian, M. L. *et al.* Outbreak of H7N8 Low Pathogenic Avian Influenza in Commercial Turkeys with Spontaneous Mutation to Highly Pathogenic Avian Influenza. *Genome Announc* **4**, https://doi.org/10.1128/genomeA.00457-16 (2016).
16. Lee, D. H., Torchetti, M. K., Killian, M. L., Berhane, Y. & Swayne, D. E. Highly Pathogenic Avian Influenza A(H7N9) Virus, Tennessee, USA, March 2017. *Emerg Infect Dis* **23**, https://doi.org/10.3201/eid2311.171013 (2017).
17. Chen, J. *et al.* First genome report and analysis of chicken H7N9 influenza viruses with poly-basic amino acids insertion in the hemagglutinin cleavage site. *Sci Rep* **7**, 9972, https://doi.org/10.1038/s41598-017-10605-6 (2017).
18. Starick, E. *et al.* Analysis of influenza A viruses of subtype H1 from wild birds, turkeys and pigs in Germany reveals interspecies transmission events. *Influenza Other Respir Viruses* **5**, 276–284, https://doi.org/10.1111/j.1750-2659.2011.00201.x (2011).
19. Yassine, H. M., Al-Natour, M. Q., Lee, C. W. & Saif, Y. M. Interspecies and intraspecies transmission of triple reassortant H3N2 influenza A viruses. *Virol J* **4**, 129, https://doi.org/10.1186/1743-422X-4-129 (2007).
20. Berhane, Y. *et al.* Molecular characterization of pandemic H1N1 influenza viruses isolated from turkeys and pathogenicity of a human pH1N1 isolate in turkeys. *Avian Dis* **54**, 1275–1285, https://doi.org/10.1637/9422-061410-Reg.1 (2010).
21. Pantin-Jackwood, M. J. *et al.* Pathogenicity and Transmission of H5 and H7 Highly Pathogenic Avian Influenza Viruses in Mallards. *J Virol* **90**, 9967–9982, https://doi.org/10.1128/JVI.01165-16 (2016).
22. Cornelissen, J. B., Vervelde, L., Post, J. & Rebel, J. M. Differences in highly pathogenic avian influenza viral pathogenesis and associated early inflammatory response in chickens and ducks. *Avian Pathol* **42**, 347–364, https://doi.org/10.1080/03079457.2013.807325 (2013).
23. Wood, G. W., Parsons, G. & Alexander, D. J. Replication of influenza A viruses of high and low pathogenicity for chickens at different sites in chickens and ducks following intranasal inoculation. *Avian Pathol* **24**, 545–551, https://doi.org/10.1080/03079459508419093 (1995).
24. Forman, A. J., Parsonson, I. M. & Doughty, W. J. The pathogenicity of an avian influenza virus isolated in Victoria. *Aust Vet J* **63**, 294–296 (1986).
25. Alexander, D. J., Allan, W. H., Parsons, D. G. & Parsons, G. The pathogenicity of four avian influenza viruses for fowls, turkeys and ducks. *Res Vet Sci* **24**, 242–247 (1978).
26. Westbury, H. A., Turner, A. J. & Kovesdy, L. Pathogenicity of 3 Australian Fowl Plague Viruses for Chickens, Turkeys and Ducks. *Vet Microbiol* **4**, 223–234, https://doi.org/10.1016/0378-1135(79)90058-0 (1979).
27. Capua, I. & Mutinelli, F. Mortality in Muscovy ducks (Cairina moschata) and domestic geese (Anser anser var. domestica) associated with natural infection with a highly pathogenic avian influenza virus of H7N1 subtype. *Avian Pathol* **30**, 179–183, https://doi.org/10.1080/03079450120044597 (2001).
28. Pantin-Jackwood, M., Swayne, D. E., Smith, D. & Shepherd, E. Effect of species, breed and route of virus inoculation on the pathogenicity of H5N1 highly pathogenic influenza (HPAI) viruses in domestic ducks. *Vet Res* **44**, 62, https://doi.org/10.1186/1297-9716-44-62 (2013).
29. Cagle, C. *et al.* Pekin and Muscovy ducks respond differently to vaccination with a H5N1 highly pathogenic avian influenza (HPAI) commercial inactivated vaccine. *Vaccine* **29**, 6549–6557, https://doi.org/10.1016/j.vaccine.2011.07.004 (2011).
30. Cagle, C. *et al.* Differences in pathogenicity, response to vaccination, and innate immune responses in different types of ducks infected with a virulent H5N1 highly pathogenic avian influenza virus from Vietnam. *Avian Dis* **56**, 479–487, https://doi.org/10.1637/10030-120511-Reg.1 (2012).
31. Salaheldin, A. H. *et al.* Potential Biological and Climatic Factors That Influence the Incidence and Persistence of Highly Pathogenic H5N1 Avian Influenza Virus in Egypt. *Front Microbiol* **9**, 528, https://doi.org/10.3389/fmicb.2018.00528 (2018).
32. Steensels, M. *et al.* Prime-boost vaccination with a fowlpox vector and an inactivated avian influenza vaccine is highly immunogenic in Pekin ducks challenged with Asian H5N1 HPAI. *Vaccine* **27**, 646–654, https://doi.org/10.1016/j.vaccine.2008.11.044 (2009).

33. Song, J. *et al.* The PA protein directly contributes to the virulence of H5N1 avian influenza viruses in domestic ducks. *J Virol* **85**, 2180–2188, https://doi.org/10.1128/JVI.01975-10 (2011).
34. Hu, J. *et al.* The PA and HA gene-mediated high viral load and intense innate immune response in the brain contribute to the high pathogenicity of H5N1 avian influenza virus in mallard ducks. *J Virol* **87**, 11063–11075, https://doi.org/10.1128/JVI.00760-13 (2013).
35. Sarmento, L., Wasilenko, J. & Pantin-Jackwood, M. The effects of NS gene exchange on the pathogenicity of H5N1 HPAI viruses in ducks. *Avian Dis* **54**, 532–537, https://doi.org/10.1637/8917-050409-Reg.1 (2010).
36. Nao, N. *et al.* A Single Amino Acid in the M1 Protein Responsible for the Different Pathogenic Potentials of H5N1 Highly Pathogenic Avian Influenza Virus Strains. *PLoS One* **10**, e0137989, https://doi.org/10.1371/journal.pone.0137989 (2015).
37. Dietze, K. *et al.* From low to high pathogenicity-Characterization of H7N7 avian influenza viruses in two epidemiologically linked outbreaks. *Transbound Emerg Dis*, https://doi.org/10.1111/tbed.12906 (2018).
38. Berhane, Y. *et al.* Highly pathogenic avian influenza virus A (H7N3) in domestic poultry, Saskatchewan, Canada, 2007. *Emerg Infect Dis* **15**, 1492–1495, https://doi.org/10.3201/eid1509.080231 (2009).
39. Abdelwhab, E. M., Veits, J. & Mettenleiter, T. C. Prevalence and control of H7 avian influenza viruses in birds and humans. *Epidemiology and infection* **142**, 896–920, https://doi.org/10.1017/S0950268813003324 (2014).
40. Londt, B. Z., Banks, J. & Alexander, D. J. Highly pathogenic avian influenza viruses with low virulence for chickens in *in vivo* tests. *Avian Pathol* **36**, 347–350, https://doi.org/10.1080/03079450701589134 (2007).
41. Horimoto, T. *et al.* Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. *Virology* **213**, 223–230, https://doi.org/10.1006/viro.1995.1562 (1995).
42. Webster, R. G., Kawaoka, Y. & Bean, W. J. Jr. Molecular changes in A/Chicken/Pennsylvania/83 (H5N2) influenza virus associated with acquisition of virulence. *Virology* **149**, 165–173 (1986).
43. Pantin-Jackwood, M. J., Stephens, C. B., Bertran, K., Swayne, D. E. & Spackman, E. The pathogenesis of H7N8 low and highly pathogenic avian influenza viruses from the United States 2016 outbreak in chickens, turkeys and mallards. *PLoS One* **12**, e0177265, https://doi.org/10.1371/journal.pone.0177265 (2017).
44. Spackman, E. *et al.* The pathogenesis of low pathogenicity H7 avian influenza viruses in chickens, ducks and turkeys. *Virol J* **7**, 331, https://doi.org/10.1186/1743-422X-7-331 (2010).
45. Tsai, H. J., Saif, Y. M., Nestor, K. E., Emmerson, D. A. & Patterson, R. A. Genetic variation in resistance of turkeys to experimental infection with Newcastle disease virus. *Avian Dis* **36**, 561–565 (1992).
46. Fuchs, W. *et al.* Molecular biology of avian infectious laryngotracheitis virus. *Vet Res* **38**, 261–279, https://doi.org/10.1051/vetres:200657 (2007).
47. Jackwood, D. J. *et al.* Identification and pathogenicity of a natural reassortant between a very virulent serotype 1 infectious bursal disease virus (IBDV) and a serotype 2 IBDV. *Virology* **420**, 98–105, https://doi.org/10.1016/j.virol.2011.08.023 (2011).
48. Pantin-Jackwood, M. J. *et al.* Role of poultry in the spread of novel H7N9 influenza virus in China. *J Virol* **88**, 5381–5390, https://doi.org/10.1128/JVI.03689-13 (2014).
49. Webster, R. G., Yakhno, M., Hinshaw, V. S., Bean, W. J. & Murti, K. G. Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology* **84**, 268–278 (1978).
50. Slemons, R. D. & Easterday, B. C. Virus replication in the digestive tract of ducks exposed by aerosol to type-A influenza. *Avian Dis* **22**, 367–377 (1978).
51. Schmolke, M. *et al.* Differential contribution of PB1-F2 to the virulence of highly pathogenic H5N1 influenza A virus in mammalian and avian species. *PLoS Pathog* **7**, e1002186, https://doi.org/10.1371/journal.ppat.1002186 (2011).
52. Hulse-Post, D. J. *et al.* Molecular changes in the polymerase genes (PA and PB1) associated with high pathogenicity of H5N1 influenza virus in mallard ducks. *J Virol* **81**, 8515–8524, https://doi.org/10.1128/JVI.00435-07 (2007).
53. Smith, J. *et al.* A comparative analysis of host responses to avian influenza infection in ducks and chickens highlights a role for the interferon-induced transmembrane proteins in viral resistance. *BMC genomics* **16**, 574, https://doi.org/10.1186/s12864-015-1778-8 (2015).
54. Barber, M. R., Aldridge, J. R. Jr., Webster, R. G. & Magor, K. E. Association of RIG-I with innate immunity of ducks to influenza. *Proc Natl Acad Sci USA* **107**, 5913–5918, https://doi.org/10.1073/pnas.1001755107 (2010).
55. Choi, J. W. *et al.* Optimal conditions for cryopreservation of primary chicken embryo kidney cells with dimethyl sulfoxide. *Mol Biotechnol* **35**, 237–241 (2007).
56. OIE. Chapter 2.3.4. — Avian influenza. Available at, http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/2.03.04_AI.pdf (2015).
57. Stech, J. *et al.* Rapid and reliable universal cloning of influenza A virus genes by target-primed plasmid amplification. *Nucleic Acids Res* **36**, e139, https://doi.org/10.1093/nar/gkn646 (2008).
58. Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G. & Webster, R. G. A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc Natl Acad Sci USA* **97**, 6108–6113, https://doi.org/10.1073/pnas.100133697 (2000).
59. Liu, Z. Q., Mahmood, T. & Yang, P. C. Western blot: technique, theory and trouble shooting. *N Am J Med Sci* **6**, 160, https://doi.org/10.4103/1947-2714.128482 (2014).
60. Hoffmann, B., Hoffmann, D., Henritzi, D., Beer, M. & Harder, T. C. Riems influenza a typing array (RITA): An RT-qPCR-based low density array for subtyping avian and mammalian influenza a viruses. *Sci Rep* **6**, 27211, https://doi.org/10.1038/srep27211 (2016).
61. Klopfleisch, R., Werner, O., Mundt, E., Harder, T. & Teifke, J. P. Neurotropism of highly pathogenic avian influenza virus A/chicken/Indonesia/2003 (H5N1) in experimentally infected pigeons (Columbia livia f. domestica). *Vet Pathol* **43**, 463–470, https://doi.org/10.1354/vp.43-4-463 (2006).
62. Abdelwhab el, S. M. *et al.* Prevalence of the C-terminal truncations of NS1 in avian influenza A viruses and effect on virulence and replication of a highly pathogenic H7N1 virus in chickens. *Virulence* **7**, 546–557, https://doi.org/10.1080/21505594.2016.1159367 (2016).

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Author Contributions

E.M.A., J.V. and T.C.M. designed the experiments and the study, D.S., A.G., M.G., A.H.S. and E.M.A carried out the animal experiments, D.S. conducted the *in-vitro* experiments, R.U. and O.I.F. performed the histopathological examination, D.S., E.M.A., T.C.H. and T.C.M. wrote the manuscript, all authors revised the manuscript.

Additional Information

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Chapter 3

Virulence of three European highly pathogenic H7N1 and H7N7 avian influenza viruses in Pekin and Muscovy ducks

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Virulence of three European highly pathogenic H7N1 and H7N7 avian influenza viruses in Pekin and Muscovy ducks

David Scheibner, Claudia Blaurock, Thomas C. Mettenleiter and Elsayed M. Abdelwhab*

Abstract

Background: There is paucity of data on the virulence of highly pathogenic (HP) avian influenza viruses (AIV) H7 in ducks compared to HPAIV H5. Here, the virulence of HPAIV H7N1 (designated H7N1-FPV34 and H7N1-It99) and H7N7 (designated H7N7-FPV27) was assessed in Pekin and/or Muscovy ducklings after intrachoanal (IC) or intramuscular (IM) infection.

Results: The morbidity rate ranged from 60 to 100% and mortality rate from 20 to 80% depending on the duck species, virus strain and/or challenge route. All Muscovy ducklings inoculated IC with H7N7-FPV27 or H7N1-FPV34 exhibited mild to severe clinical signs resulting in the death of 2/10 and 8/10 ducklings, respectively. Also, 2/10 and 6/9 of inoculated Muscovy ducklings died after IC or IM infection with H7N1-It99, respectively. Moreover, 5/10 Pekin ducklings inoculated IC or IM with H7N1-It99 died. The level of virus detected in the oropharyngeal swabs was higher than in the cloacal swabs.

Conclusion: Taken together, HPAIV H7 cause mortality and morbidity in Muscovy and Pekin ducklings. The severity of disease in Muscovy ducklings depended on the virus strain and/or route of infection. Preferential replication of the virus in the respiratory tract compared to the gut merits further investigation.

Keywords: Ducks, Muscovy duck, Pekin duck, Highly pathogenic avian influenza virus, HPAIV, H7N7, H7N1, Virulence

Background

Avian influenza viruses (AIV) belong to the family *Orthomyxoviridae*. The genome of AIV contains eight gene segments, which encode at least 11 viral proteins. They are classified according to the antigenic properties of the hemagglutinin (HA) and neuraminidase (NA) proteins into 16 HA (H1 to H16) and 9 NA (N1 to N9) subtypes [1]. Wild aquatic birds are the reservoir for all AIV and they transmit the virus to domestic birds. All AIV subtypes are low pathogenic (LP) causing mild local infection with or without overt clinical signs. Some H5 and H7 viruses can exhibit a highly pathogenic (HP) phenotype, mostly in domestic birds, causing multiorgan dysfunction due to systemic replication of the virus [2]. Wild and domestic ducks play an important role as a reservoir for AIV since the infection is usually asymptomatic. However, unlike the high mortality generally caused by HPAIV in chickens, some studies showed that the susceptibility of ducks to HPAIV differs by duck species (e.g. Pekin, Mallards or Muscovy), infection route, and/or age of ducks (i.e. ducklings are more susceptible than adult ducks) [3–9]. Muscovy ducks are more vulnerable than Pekin ducks to HPAIV H5N1 due to differences in immune responses [3, 4, 8]. However, these studies had been conducted using H5 HPAIV. In contrast, only limited data are available on the virulence of European H7 viruses, particularly in Muscovy ducks.

HPAIV H7 were first isolated in Europe in the early 1900s and have frequently been detected in poultry and wild birds in several European countries in the last two decades. Historical outbreaks caused by several H7Nx viruses in Europe in 1902, 1927, 1934, 1980s as well as recent outbreaks in 1999, 2003 and 2015 were described [10–15]. There is little information, if any, on the virulence of these H7 viruses in ducks. Neurological disorders and high mortality were observed in Muscovy

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ducks during the HPAIV H7N1 outbreaks in Italy in 1999–2000 [16]. However, experimental data from Muscovy ducks are still lacking. Three-week-old Pekin ducks did not show clinical signs, weight loss and/or mortality after the challenge with two different Italian HPAIV H7N1 [17, 18].

The objective of this study was to compare the virulence of two historic HPAIV A/FPV/Dutch/27 (H7N7) (designated hereafter as H7N7-FPV27) and A/Germany/FPV/1934 (H7N1) (designated hereafter H7N1-FPV34) and the recent HPAIV A/chicken/Italy/445/1999 (H7N1) (designated hereafter H7N1-It99) in Muscovy ducks (*Cairina moschata*) after intrachoanal (IC) inoculation. Furthermore, the impact of route of infection and species of ducks on virulence of H7N1-It99 was studied in Muscovy ducks and Pekin ducks (*Anas platyrhynchos domesticus*) after IC or intramuscular (IM) infection. The IC route was recommended for studying the pathogenesis of AIV to simulate natural upper respiratory exposure/transmission and to ensure that each bird receives the full dose [19, 20]. Likewise, IM injection was recently used to assess the pathogenicity of HPAIV in ducklings, resembling intravenous pathogenicity index (IVPI) in chickens [9]. Previous studies have shown that all three viruses were highly lethal in chickens under experimental conditions [10, 21, 22].

Results

In this study, the virulence of the two historic H7N7-FPV27 (group 1) and H7N1-FPV34 (group 2) in Muscovy ducks was assessed. The impact of duck species and inoculation route on virulence of H7N1-It99 was tested in Muscovy (groups 3 and 4) and Pekin ducks (groups 5 and 6) after IC or IM infection. All birds were observed for 10 days and clinically scored as 0 (healthy), 1 (sick), 2 (severely sick) or 3 (dead) and the pathogenicity index (PI) was calculated as a scale from 0 (avirulent) to 3 (highly virulent) [23].

Clinical examination

After challenge, Muscovy ducks showed diarrhea and nervous signs including circling, rolling, incoordination, steady gait and/or opisthotonus starting from 2 dpi. The morbidity rate ranged from 60 to 100% and mortality from 20 to 80% (Fig. 1a and b).

All Muscovy ducklings inoculated with H7N7-FPV27 (group 1) showed mild depression at 1 dpi, which became more prominent at 2 dpi. All ducklings in this group returned to normal starting from 3 dpi except two ducklings that died or were killed for humane reasons by day 7 (Table 1) after showing severe depression and central nervous signs. Both birds were scored dead at 8 dpi. The PI value was 0.8 (Table 1).

Muscovy ducklings challenged with H7N7-FPV34 (group 2) exhibited significantly ($p < 0.01$) more severe and prominent signs than ducklings in group 1 and group 3 with a PI of 1.8 (Table 1). At 3 dpi, two ducklings showed moderate nervous signs. At 4 dpi, one bird died and four birds were humanely killed due to severe nervous signs and scored as dead at 5 dpi. Likewise, at 6 dpi, two ducklings died and another duckling was killed and scored dead at 7 dpi. In total, eight out of ten ducklings died between 4 and 7 dpi with MDT of 5.4 days. Two birds survived, however showing mild to moderate clinical signs at 10 dpi (Fig. 1a and b).

Seven out of ten Muscovy ducklings inoculated IC with H7N1-It99 (group 3) showed neurological signs, while three ducklings remained healthy to the end of the experiment (PI of 0.5). Clinical signs started at 4 dpi. One bird showed mild to severe clinical signs from 4 dpi to 10 dpi. Meanwhile, four birds showed mild transient depression and recovered by 8 dpi. Two ducklings were killed at 5 and 6 dpi (and scored dead at the next day) for humane reasons after showing severe clinical signs (MDT = 6.5) (Table 1, Fig. 1a and b).

Muscovy ducklings inoculated IM with H7N1-It99 (group 4) showed higher morbidity and mortality

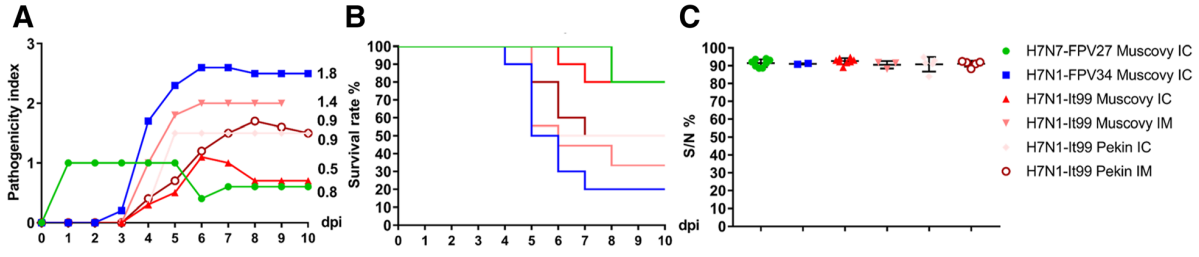

Fig. 1 Pathogenicity index, survival rate and seroconversion in Muscovy and Pekin ducks after challenge with H7 viruses. Shown are the average clinical scores for each group. All birds were observed for 10 days and clinically scored according to the OIE recommendations as 0 (healthy), 1 (sick), 2 (severely sick) or 3 (dead) and the pathogenicity index (PI) was calculated as a scale from 0 (avirulent) to 3 (highly virulent). The PI, shown as numbers, was calculated by dividing the sum of the arithmetic mean values of daily scores by 10 (the number of observation days). Dead birds were score 3 until the end of the experiment according to the OIE recommendations (**a**). Survival rate in each group showing the daily mortality of ducks after infection (**b**) and antibody titers at the end of experiment using ELISA (expressed as mean ± standard deviation) (**c**)

Table 1 Clinical examination of Muscovy and Pekin ducks after challenge with H7 viruses

Group	Species	Challenge route	Virus	Morbidity (%)	Mortality (%)	MDT (range)	PI
1	Muscovy	IC	H7N7-FPV27	10/10 (100%)	2/10 (20%)	7 (7)	0.8
2	Muscovy	IC	H7N1-FPV34	10/10 (100%)	8/10 (80%)	5.4 (4–7)	1.8
3	Muscovy	IC	H7N1-It99	7/10 (70%)	2/10 (20%)	6.5 (6–7)	0.5
4	Muscovy	IM	H7N1- It99	9/9 (100%)	6/9 (66.7%)	5.7 (5–8)	1.4
5	Pekin	IC	H7N1- It99	6/10 (60%)	5/10 (50%)	5 (5)	0.9
6	Pekin	IM	H7N1- It99	8/10 (80%)	5/10 (50%)	6 (5–7)	0.9

Range refers to the day of first and last mortality
IC intrachoanal, *IM* intramuscular, *MDT* mean death time per day calculated for dead birds, *PI* Pathogenicity index

compared to IC-inoculated animals (group 3). In total, six out of nine Muscovy ducklings inoculated IM with H7N1-It99 died with a PI of 1.4 and MDT of 5.7 days: three ducklings were killed at 4 dpi and one at 7 dpi. Two birds were found dead at 5 and 6 dpi. Two ducklings remained sick until 10 dpi and one bird recovered at 9 dpi (Fig. 1a and b).

Six out of ten Pekin ducklings inoculated IC with H7N1-It99 (group 5) showed clinical signs (PI 0.9) where five Pekin ducklings died at 5 dpi (Fig. 1a and b) with or without showing moderate depression and nervous signs. One bird had temporary torticollis; however, it accessed food and water easily until 10 dpi. The other four ducklings did not show any clinical signs.

Eight out of ten Pekin ducklings injected IM with H7N1-It99 (group 6) showed clinical signs (PI = 0.9) where five Pekin ducklings died at 5 dpi (n = 2), day 6 dpi (n = 2) and 7 dpi (n = 1) after showing mild to moderate nervous signs (Fig. 1a and b). Three ducklings showed transient mild to moderate depression two of which recovered after 2 days. Two ducklings did not show any clinical signs during the observation period. No statistical difference in clinical scoring of IM or IC inoculated ducks was observed ($P > 0.99$).

Virus shedding

Influenza virus RNA was not detected in swab samples collected before infection. Cloacal (CL) and oropharyngeal (OP) swabs collected from inoculated birds at 2, 4, 7 and 10 dpi were tested by real time RT-PCR. Results are summarized in Figs. 2 and 3. Swab samples collected at 10 dpi were negative (data not shown).

IC-inoculated Muscovy ducks (groups 1, 2 and 3)

Virus excretion in the OP swabs was higher than in CL swabs particularly at 2 and 4 dpi (Fig. 2). The mean quantity of virus excreted in the OP and CL swabs at 4 dpi was higher than the quantity of virus excretion at 2 and 7 dpi (Fig. 2). At 2 dpi, the virus was detected in OP swabs in all ducks, except 2 Muscovy ducks inoculated

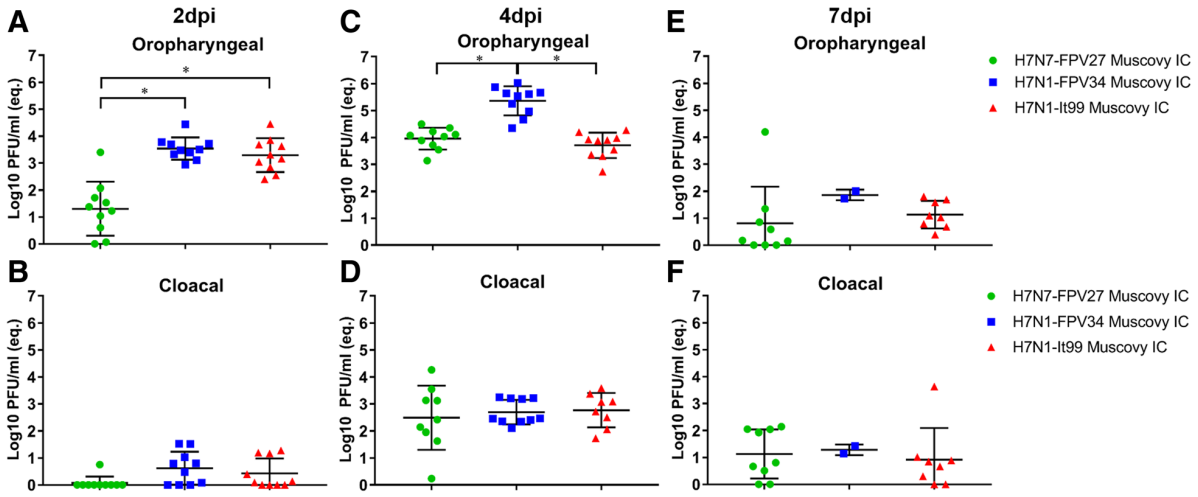

Fig. 2 Virus excretion in oropharyngeal and cloacal swabs after intrachoanal inoculation of Muscovy ducks with HPAIV H7 viruses. Virus excretion in oropharyngeal and cloacal swabs at 2 (**a**-**b**), 4 (**c**-**d**) and 7 (**e**-**f**) days post inoculation using RT-qPCR expressed by equivalent Log10 PFU/mL. All results are expressed as average and standard deviation (mean ± SD) per group at indicated time points post infection. The viruses were not detected in swabs collected at 10 dpi (data not shown). Statistical significance shown in asterisks indicate P values < 0.05

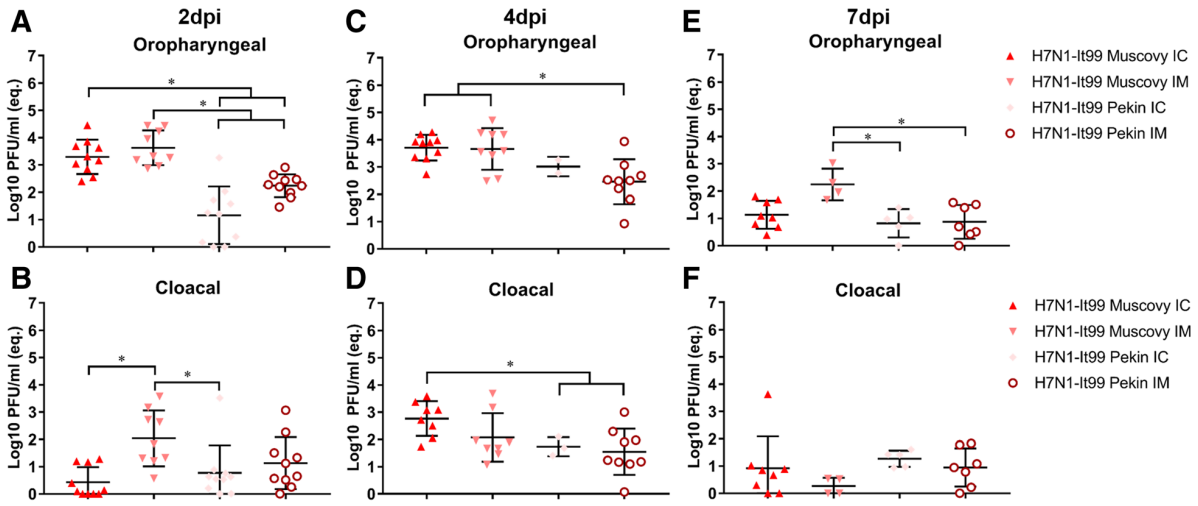

Fig. 3 Virus excretion in the oropharyngeal and cloacal swabs in Muscovy and Pekin ducks challenged with H7N1-It99 by intrachoanal or intramuscular routes. Virus excretion in oropharyngeal and cloacal swabs at 2 (**a**-**b**), 4 (**c**-**d**) and 7 (**e**-**f**) days post inoculation using RT-qPCR expressed by equivalent Log10 PFU/mL. All results are expressed as average and standard deviation (mean ± SD) per group at indicated time points post infection. The viruses were not detected in swabs collected at 10 dpi (data not shown). Statistical significance shown in asterisks indicate *P* values < 0.05. For clarity, results of H7N1-It99 IC-inoculated ducks (group 3) was presented in Figure 3 again for comparison

with H7N7-FPV27 (group 1) (Fig. 2a). The latter virus was excreted in OP swabs at significantly lower amounts than H7N1-FPV34 (group 2) and H7N1-It99 (group 3) ($P < 0.001$) at 2 dpi (Fig. 2a). At 4 dpi, H7N1-FPV34 was shed with significantly higher amounts in the OP swabs than the other two viruses (Fig. 2c), while at 7 dpi (Fig. 2e) and in CL swabs at each time all viruses were excreted at comparably similar levels (Fig. 2b, d and f).

Muscovy ducks (groups 3 and 4) and Pekin ducks (groups 5 and 6) challenged with H7N1-It99

The mean quantity of virus excretion in the OP swabs was higher than in CL swabs particularly at 2 and 4 dpi regardless of duck species or inoculation route (Fig. 3a-d). In the OP swabs, there was no significant difference in the amount of H7N1-It99 excreted from Muscovy (groups 3 and 4) or Pekin ducklings (groups 5 and 6) after IM or IC challenge at each time point. The mean quantity of virus excreted in the OP swabs by Muscovy ducks was higher than the virus excreted by Pekin ducks (Fig. 3).

Seroconversion

All serum samples collected before infection were negative for anti-influenza antibodies. At the end of the experiment, all surviving birds possessed anti-NP antibodies detectable by ELISA. There was no significant difference in antibody titers between the different groups of ducklings regardless of the virus subtype, species or inoculation route (Fig. 1c).

Discussion

Ducks play an important role as a reservoir for AIV including HPAIV [8, 24]. However, data on the susceptibility of Pekin ducks are scarce and no data on the susceptibility of Muscovy ducks to H7 viruses particularly from Europe are available. In Muscovy ducklings, the historic H7N1-FPV34 was more virulent than historic H7N7-FPV27 and contemporary H7N1-It99 after IC inoculation as indicated by higher morbidity and mortality. This is partially in agreement with findings in a previous historic study [10] which showed that 9/10 intranasally inoculated two-week-old Khaki Campbell ducklings, an egg-laying duck breed, with H7N1-FPV34 exhibited clinical signs and 2/10 ducklings died at 7 and 11 dpi while H7N7-FPV27 did not cause any clinical signs or mortality [10].

Muscovy ducklings infected with H7N7-It99 showed more severe symptoms than IC inoculated animals, while no impact of the infection route on virulence was observed in Pekin ducklings. In a previous study, intranasal, intrachoanal or ocular infection with an HPAIV H5N1 produced similar outcome in two-week-old Pekin or Muscovy ducks with an HPAIV H5N1 [8]. C Grund, et al. [9] reported higher mortality in Pekin and Muscovy ducks after IM injection than oculonasal inoculation with HPAIV H5N8. Mortality in Mallard ducks after intravenous injection was strain-dependent (i.e. some HPAIV H5N1 induced 100% mortality while others were avirulent) [25].

In the current study, five of ten Pekin ducklings died after IC or IM infection with H7N1-It99, whereas

inoculation of three-week-old Pekin ducks with two different Italian HPAIV H7N1 induced no mortality [17, 18]. Here, we used ten-day-old ducklings because it has been shown that younger ducklings are more susceptible to some HPAIV H5N1 than adult ducks [26, 27]. Differences in the age of Pekin ducks as well as the use of different virus strains may explain the higher mortality in Pekin ducks in the current study compared to previous studies using HPAIV H5 [17, 18, 26].

Muscovy ducks are more vulnerable than Pekin ducks to some H5N1 viruses [6, 8, 9]. On the other hand, mortality was observed only in Pekin but not in Muscovy ducklings after challenge with an HPAIV H5N1 [7]. So far, no data are available on the susceptibility of Muscovy ducks to HPAIV H7. Recently, we have shown that a German HPAIV H7N7 did not result in any mortality in Pekin or in Muscovy ducks (Scheibner et al. submitted). In the current study, H7N1-It99 induced a lower mortality rate in Muscovy ducklings inoculated IC (20%) than Pekin ducklings inoculated by the same route (50%). Interestingly, Muscovy ducklings excreted H7N1-It99 virus at significantly higher levels than Pekin ducklings indicating important role in spreading of the virus into the environment. Therefore, it is important to consider subtype/strain variations in the assessment of virulence of HPAIV H7 in different duck species.

AIV preferentially replicate in the digestive tract of ducks, which may enable continuous shedding of the virus into the environment (i.e. water ponds) [28–30]. Interestingly, our results indicated that although all viruses were excreted in OP and CL swabs at 2, 4 and 7 dpi, the amount in OP swabs was higher than in CL swabs regardless of the challenge virus, duck species or route of infection. Similar results were observed after experimental infection of Mallard ducks with HPAIV Tk/Italy/99 (H7N1), Ck/Netherlands/03 (H7N7), Ck/North Korea/05 (H7N7) and Ck/Victoria/85 (H7N7). Conversely, HPAIV Ck/Jalisco/12 (H7N3) and Ck/Canada/05 (H7N3) were excreted in a higher amount in the CL than in OP swabs [24]. The preferential pattern of virus excretion of H7 viruses from the oropharynx merits further investigation.

Conclusions

Taken together, the three European H7 viruses used in this study exhibited variable virulence in Muscovy ducklings. H7N1-FPV34 induced 80% mortality, while H7N7-FPV27 and H7N1-It99 killed only 20% of IC-inoculated ducklings. H7N1-It99 exhibited higher virulence in IM-injected Muscovy than in IC inoculated ducklings with 66.7% and 20 mortality, respectively. Furthermore, H7N1-It99 exhibited moderate virulence in Pekin ducklings with no difference between the IC or IM inoculation routes. Moreover, regardless of the challenge route, Muscovy ducks excreted higher amounts of H7N1-It99 than Pekin ducks. Findings in this study showed the variable virulence of HPAIV H7 in different duck species.

Methods

The main goal of this study was to assess the virulence of three European H7 viruses in domestic ducks. Muscovy and Pekin ducklings were inoculated via the intrachoanal and/or intramuscular and were observed for 10 days post inoculation/injection (dpi). Swab samples were collected from all ducklings at 2, 4, 7 and 10 dpi and tested by generic real time RT-PCR.

Virus propagation

Viruses in this study were kindly provided by Timm C. Harder. All viruses were propagated in the allantoic sac of specific pathogen free (SPF) embryonated chicken eggs (ECE) (VALO BioMedia GmbH) according to the standard protocol [23]. Allantoic fluid was collected and the hemagglutination activity was measured using 1% chicken erythrocytes [23]. Aliquots of virus stocks were kept at − 70 °C until use. All viruses were propagated and handled in biosafety level 3 laboratory at the FLI.

Virus titration

Virus titration was done using plaque assay. Confluent MDCKII cells in 12-well plates were infected with ten-fold serial dilutions of specified viruses for an hour at 37 °C/5% CO_2. Cells were overlaid with semisolid BactoTM Agar (BD) containing minimal essential medium (MEM) and 4% bovine serum albumin (BSA) (MP Biomedicals). All plates were incubated for 3 days at 37 °C. Cells were fixed by 10% formaldehyde containing 0.1% crystal violet. Plaques were counted and viral titers were expressed as plaque forming units per ml (PFU/ml).

Animal experiment

Animal experiments were carried out after approval by the authorized ethics committee of the State Office of Agriculture, Food Safety, and Fishery in Mecklenburg – Western Pomerania (No. 7221.3-1-060/17) and approval by the commissioner for animal welfare at the FLI representing the Institutional Animal Care and Use Committee (IACUC) following the German Regulations for Animal Welfare.

One-day old commercial Pekin and Muscovy ducklings were purchased from Czarkowski GbR, Storkow, Germany. At the FLI, swab samples were collected from all ducks and examined to exclude infection by influenza [31] and Salmonella spp. [32, 33]. Birds were housed in different groups and food and water were added ad-libitum. At 10 days of age, male and female Pekin and Muscovy ducklings were randomly allocated to separate

groups. At day 0, ten birds were inoculated with 0.2 mL containing 5×10^5 PFU via the IC or IM route as described in Table 1. All animals were observed for 10 days and clinically scored [21]. Briefly, healthy ducks were given score (0), sick birds showing one clinical sign (e.g. depression, diarrhea, nervous manifestations, respiratory disorders) were given score (1), severely sick birds showed more than one clinical sign were given score (2) and dead birds were given score (3). The pathogenicity index (PI) was calculated by dividing the sum of the arithmetic mean values of daily scores by 10 (the number of observation days). The PI for each virus ranged from 0 (avirulent) to 3 (highly virulent) [23]. Serum and swab samples were collected and stored in BSL-3 laboratories, at – 20° and – 70 °C, respectively.

Virus excretion

OP and CL swabs were collected before infection and at 2, 4, 7 and 10 days post inoculation/injection (dpi) on swab media and stored at – 70 °C until use. Swabs medium contained (pro liter) MEM Eagle (Sigma-Aldrich), 5.6 mL BSA (MP Biomedicals) and antibiotics (1% enrofloxacin, 0.5% lincomycin and 0.1% gentamycin). The RNA was extracted from swab media using NucleoMagVet® 8/96 PCR Clean-up Core Kit (Macherey & Nagel GmbH, Germany) in KingFisher Flex Purification System (Thermo Fisher Scientific, USA). The quantity of virus excretion in swab samples was determined using SuperScript™ III Platinum™ One-Step qRT-PCR Kit (Invitrogen, Germany) according to the manufacturer guidelines and generic real-time reverse-transcription polymerase chain reaction (RT-qPCR) [31]. The RT-qPCR reactions were performed in AriaMx Real-time PCR System (Agilent, Germany). For RT-PCR amplification the following thermal profile was applied: 30 min at 50 °C (reverse transcription) and 15 min at 94 °C (inactivation of the reverse transcriptase/activation Taq polymerase), followed by 42 cycles at 94 °C for 30s (denaturation), 58 °C for 30s (annealing) and 68 °C for 30s (elongation). Standard curves using HPAIV H7N7 (10^1 to 10^6 pfu/mL) were run in each RT-qPCR round. The relative amount of excreted virus was quantified by plotting the Ct-values in the standard curves and results are expressed as average ± standard deviation equivalent $\log_{10}$ PFU/ml.

Serological examination

Blood was collected before infection (5 samples pro species) via wing vein puncture and at the end of the experiments from all surviving ducks after euthanization using isoflurane® (CP-Pharma). Briefly, the ducklings were gently and carefully put inside a tightly close beaker containing four to five isoflurane-soaked gauze sponges for about 2 min to ensure deep anesthesia. Complete loss of consciousness was achieved as assessed by complete suppression of pedal and ocular reflexes. Ducklings were taken out and whole blood was collected in 50 mL Falcon tubes via cutting the jugular vein using knife. Thereafter, the head was separated from the body rapidly and completely. The serum was separated from the blood after 24 h incubation in the fridge followed by centrifugation and inactivation at 72 °*C. sera* were tested for anti-AIV nucleoprotein (NP) using enzyme-linked immunosorbent assay (ELISA) by ID screen Influenza A Antibody Competition Multispecies kit (IDvet). Plates were read in Tecan® ELISA reader. The cut-off point according to the manufacture guideline was 55%, samples between 45 and 55% were considered questionable and samples lower than 45% were considered negative.

Statistics

Statistical differences were analyzed using non-parametric Kruskal-Wallis and Mann-Whitney Wilcoxon tests with post hoc Tukey tests. Results were considered statistically significant by any test at p value < 0.05. All analysis was done by GraphPad Prism software. Clinical scoring for mean values for each bird in 10 day-observation period was compared.

Abbreviations

AIV: Avian influenza viruses; BSA: Bovine serum albumin; CL: Cloacal; dpi: Days post inoculation/injection; ECE: Embryonated chicken eggs; ELISA: Enzyme linked immunosorbent assay; FLI: Friedrich-Loeffler-Institut; HA: Hemagglutinin; HP: Highly pathogenic; IACUC: Institutional Animal Care and Use Committee; IC: Intrachoanal; IM: Intramuscular; IVPI: Intravenous pathogenicity index; LP: Low pathogenic; MDCKII: Madin-Darby canine kidney cells type II; MDT: Mean death time; MEM: Minimum essential medium; NA: Neuraminidase; NP: Nucleoprotein; OP: Oropharyngeal; PCR: Polymerase chain reaction; PFU/mL: Plaque forming units per ml; RT-qPCR: Real-time reverse-transcription polymerase chain reaction; SPF: Specific pathogen free

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Conceptualization, EMA and TCM; Methodology, DS, CB and EMA; Software, DS; Validation, DS and EMA; Formal Analysis, DS and EMA; Investigation, DS, CB and EMA; Resources, EMA and TCM; Data Curation, DS and EMA; Writing – Original Draft Preparation, DS and EMA; Writing – Review & Editing, DS, EMA and TCM; Visualization, DS, EMA and TCM; Supervision, EMA and TCM;

Project Administration, EMA and TCM; Funding Acquisition, EMA and TCM. All authors have read and approved the manuscript.

Ethics approval and consent to participate
All animal trials have been approved by the authorized ethics committee of the State Office of Agriculture, Food Safety, and Fishery in Mecklenburg – Western Pomerania (No. 7221.3-1-060/17) and by the commissioner for animal welfare at the FLI representing the Institutional Animal Care and Use Committee (IACUC) following the German Regulations for Animal Welfare.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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References

1. Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol. 2005;79(5):2814–22.
2. Alexander DJ. A review of avian influenza in different bird species. Vet Microbiol. 2000;74(1–2):3–13.
3. Cagle C, Wasilenko J, Adams SC, Cardona CJ, To TL, Nguyen T, Spackman E, Suarez DL, Smith D, Shepherd E, et al. Differences in pathogenicity, response to vaccination, and innate immune responses in different types of ducks infected with a virulent H5N1 highly pathogenic avian influenza virus from Vietnam. Avian Dis. 2012;56(3):479–87.
4. Cagle C, To TL, Nguyen T, Wasilenko J, Adams SC, Cardona CJ, Spackman E, Suarez DL, Pantin-Jackwood MJ. Pekin and Muscovy ducks respond differently to vaccination with a H5N1 highly pathogenic avian influenza (HPAI) commercial inactivated vaccine. Vaccine. 2011;29(38):6549–57.
5. Wei L, Jiao P, Song Y, Cao L, Yuan R, Gong L, Cui J, Zhang S, Qi W, Yang S, et al. Host immune responses of ducks infected with H5N1 highly pathogenic avian influenza viruses of different pathogenicities. Vet Microbiol. 2013;166(3–4):386–93.
6. Salaheldin AH, Kasbohm E, El-Naggar H, Ulrich R, Scheibner D, Gischke M, Hassan MK, Arafa AA, Hassan WM, Abd El-Hamid HS, et al. Potential biological and climatic factors that influence the incidence and persistence of highly pathogenic H5N1 avian influenza virus in Egypt. Front Microbiol. 2018;9:528.
7. Steensels M, Bublot M, Van Borm S, De Vriese J, Lambrecht B, Richard-Mazet A, Chanavat-Bizzini S, Duboeuf M, Le Gros FX, Van den Berg T. Prime-boost vaccination with a fowlpox vector and an inactivated avian influenza vaccine is highly immunogenic in Pekin ducks challenged with Asian H5N1 HPAI. Vaccine. 2009;27(5):646–54.
8. Pantin-Jackwood M, Swayne DE, Smith D, Shepherd E. Effect of species, breed and route of virus inoculation on the pathogenicity of H5N1 highly pathogenic influenza (HPAI) viruses in domestic ducks. Vet Res. 2013;44:62.
9. Grund C, Hoffmann D, Ulrich R, Naguib M, Schinkothe J, Hoffmann B, Harder T, Saenger S, Zscheppang K, Tonnies M, et al. A novel European H5N8 influenza A virus has increased virulence in ducks but low zoonotic potential. Emerg Microbes Infect. 2018;7(1):132.
10. Alexander DJ, Allan WH, Parsons DG, Parsons G. The pathogenicity of four avian influenza viruses for fowls, turkeys and ducks. Res Vet Sci. 1978;24(2):242–7.
11. Abdelwhab EM, Veits J, Mettenleiter TC. Prevalence and control of H7 avian influenza viruses in birds and humans. Epidemiol Infect. 2014;142(5):896–920.
12. Dietze K, Graaf A, Homeier-Bachmann T, Grund C, Forth L, Pohlmann A, Jeske C, Wintermann M, Beer M, Conraths FJ, et al. From low to high pathogenicity-characterization of H7N7 avian influenza viruses in two epidemiologically linked outbreaks. Transbound Emerg Dis. 2018;65:1576.
13. Seekings AH, Slomka MJ, Russell C, Howard WA, Choudhury B, Nunez A, Londt BZ, Cox W, Ceeraz V, Thoren P, et al. Direct evidence of H7N7 avian influenza virus mutation from low to high virulence on a single poultry premises during an outbreak in free range chickens in the UK, 2008. Infect Genet Evol. 2018;64:13–31.
14. Stegeman A, Bouma A, Elbers AR, de Jong MC, Nodelijk G, de Klerk F, Koch G, van Boven M. Avian influenza A virus (H7N7) epidemic in The Netherlands in 2003: course of the epidemic and effectiveness of control measures. J Infect Dis. 2004;190(12):2088–95.
15. Munster VJ, Wallensten A, Baas C, Rimmelzwaan GF, Schutten M, Olsen B, Osterhaus AD, Fouchier RA. Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe. Emerg Infect Dis. 2005;11(10):1545–51.
16. Capua I, Mutinelli F. Mortality in Muscovy ducks (Cairina moschata) and domestic geese (Anser anser var. domestica) associated with natural infection with a highly pathogenic avian influenza virus of H7N1 subtype. Avian Pathol. 2001;30(2):179–83.
17. Cornelissen JB, Vervelde L, Post J, Rebel JM. Differences in highly pathogenic avian influenza viral pathogenesis and associated early inflammatory response in chickens and ducks. Avian Pathol. 2013;42(4):347–64.
18. Aldous EW, Seekings JM, McNally A, Nili H, Fuller CM, Irvine RM, Alexander DJ, Brown IH. Infection dynamics of highly pathogenic avian influenza and virulent avian paramyxovirus type 1 viruses in chickens, turkeys and ducks. Avian Pathol. 2010;39(4):265–73.
19. Spackman E, Pantin-Jackwood M, Swayne DE, Suarez DL, Kapczynski DR. Impact of route of exposure and challenge dose on the pathogenesis of H7N9 low pathogenicity avian influenza virus in chickens. Virology. 2015;477:72–81.
20. McKinley ET, Spackman E, Pantin-Jackwood MJ. The pathogenesis of H3N8 canine influenza virus in chickens, turkeys and ducks. Influenza Other Respir Viruses. 2010;4(6):353–6.
21. Abdelwhab el SM, Veits J, Tauscher K, Ziller M, Teifke JP, Stech J, Mettenleiter TC. A unique multibasic proteolytic cleavage site and three mutations in the HA2 domain confer high virulence of H7N1 avian influenza virus in chickens. J Virol. 2015;90(1):400–11.
22. Westbury HA, Turner AJ, Kovesdy L. Pathogenicity of 3 Australian fowl plague viruses for chickens, turkeys and ducks. Vet Microbiol. 1979;4(3):223–34.
23. OIE: Chapter 2.3.4. — Avian influenza. 2015. Available at: http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.03.04_AI.pdf. Accessed 21 Nov 2018.
24. Pantin-Jackwood MJ, Costa-Hurtado M, Shepherd E, DeJesus E, Smith D, Spackman E, Kapczynski DR, Suarez DL, Stallknecht DE, Swayne DE. Pathogenicity and transmission of H5 and H7 highly pathogenic avian influenza viruses in mallards. J Virol. 2016;90(21):9967–82.
25. Tang Y, Wu P, Peng D, Wang X, Wan H, Zhang P, Long J, Zhang W, Li Y, Wang W, et al. Characterization of duck H5N1 influenza viruses with differing pathogenicity in mallard (Anas platyrhynchos) ducks. Avian Pathol. 2009;38(6):457–67.
26. Londt BZ, Nunez A, Banks J, Alexander DJ, Russell C, Richard-Londt AC, Brown IH. The effect of age on the pathogenesis of a highly pathogenic avian influenza (HPAI) H5N1 virus in Pekin ducks (Anas platyrhynchos) infected experimentally. Influenza Other Respir Viruses. 2010;4(1):17–25.
27. Pantin-Jackwood MJ, Smith DM, Wasilenko JL, Cagle C, Shepherd E, Sarmento L, Kapczynski DR, Afonso CL. Effect of age on the pathogenesis and innate immune responses in Pekin ducks infected with different H5N1 highly pathogenic avian influenza viruses. Virus Res. 2012;167(2):196–206.
28. Kida H, Yanagawa R, Matsuoka Y. Duck influenza lacking evidence of disease signs and immune response. Infect Immun. 1980;30(2):547–53.
29. Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology. 1978;84(2):268–78.
30. Slemons RD, Easterday BC. Virus replication in the digestive tract of ducks exposed by aerosol to type-A influenza. Avian Dis. 1978;22(3):367–77.
31. Hoffmann B, Hoffmann D, Henritzi D, Beer M, Harder TC. Riems influenza a typing array (RITA): an RT-qPCR-based low density array for subtyping avian and mammalian influenza a viruses. Sci Rep. 2016;6:27211.
32. Pathmanathan SG, Cardona-Castro N, Sanchez-Jimenez MM, Correa-Ochoa MM, Puthucheary SD, Thong KL. Simple and rapid detection of Salmonella strains by direct PCR amplification of the hilA gene. J Med Microbiol. 2003;52(Pt 9:773–6.
33. Halatsi K, Oikonomou I, Lambiri M, Mandilara G, Vatopoulos A, Kyriacou A. PCR detection of Salmonella spp. using primers targeting the quorum sensing gene sdiA. FEMS Microbiol Lett. 2006;259(2):201–7.

Chapter 4

General Discussion

IV

5 General Discussion

Avian influenza viruses cause significant economic losses in poultry worldwide and pose a serious public health threat (Alexander, 2000; Mostafa et al., 2018; Pantin-Jackwood and Swayne, 2009). A number of factors such as a broad host range, high mutation rates and the ability for reassortment and recombination result in fast, unpredictable and complex virus evolution (Ferguson et al., 2003). Only H5 and H7 viruses are able to evolve naturally to highly pathogenic viruses from low pathogenic ancestors (Alexander, 2000). In contrast to H5Nx viruses, the mechanism for the evolution of HPAIV H7 has not been adequately investigated.

H7 viruses, particularly H7N7 and H7N1, are very common in birds worldwide (Abdelwhab et al., 2014). In Germany, H7N7 viruses had been frequently isolated from domestic birds in the last two decades. The vast majority of reported outbreaks caused by low pathogenic AIV resulted in only mild clinical signs, if any. Nevertheless, these birds had to be culled since H5 and H7 viruses are notifiable because of their potential to shift to high virulence (Abdelwhab et al., 2014; Verbraucherschutz, 2019). Classification of AIV into LP and HP pathotypes is based on the pathogenicity of the viruses in chickens, which is the most common domestic bird species. However, virulence markers differ greatly from the species to another (e.g. chickens vs. ducks or chickens vs. mammals) (Mostafa et al., 2018; Pantin-Jackwood and Swayne, 2009). Therefore, understanding the genetic mechanism for the evolution of LP H5 and H7 into HP in different bird species is of great importance for risk assessment and implementation of effective control measures. In 2015, isolation of HPAIV H7N7 and the putative LP ancestor from the same chicken farm provided the opportunity to analyze the underlying mechanism for the evolution of HPAIV H7N7 in different poultry species (Dietze et al., 2018).

Chickens are known to be highly susceptible to AIV infections which is indicated by high mortality and morbidity after infection with HPAIV (Pantin-Jackwood and Swayne, 2009). As described in the first study by **Scheibner et al. (2019b)**, LPAIV H7N7 was avirulent in chickens, excreted in oropharyngeal and cloacal swabs and tissue tropism was limited mainly to the spleen and intestinal tracts. This is in accordance with the common knowledge about LPAIV infection in chickens (Pantin-Jackwood and Swayne, 2007). However, multiorgan tropism of a Chinese H9N2 virus to lung, trachea, kidney and bursa of Fabricius leading to death has also been reported in immunocompromised chickens because of secondary infections with bacteria (Bano et al., 2003; Li et al., 2003). Conversely, HPAIV H7N7 caused 100% mortality in both inoculated and sentinel chickens and the NP antigen was detected in the parenchyma and endothelial cells of all organs. Insertion of the polybasic CS (designated LP-Poly) increased virulence to levels that were only slightly lower than that of the HP after oculonasal inoculation and the virus was detectable in most of the examined organs. Previous studies have also shown that the polybasic HACS is the main virulence determinant in some HPAIV H5Nx and

H7N7 in chickens (Abdelwhab et al., 2016; Kobayashi et al., 1996). However, Abdelwhab el et al. (2015) showed that the polybasic HACS as virulence marker is virus-specific. The German LP H7N7 viruses from 2001, 2003 and 2013 with polybasic HACS were as virulent and transmissible as HPAIV H7N7. In contrast, H7N7 from 2001 and the Italian H7N1 viruses required additional mutations in the HA or other gene segments to confer high virulence in chickens. Furthermore some H5 viruses required, in addition to the polybasic HACS, mutations in the NA (Stech et al., 2015), NS1 (Chen et al., 2013) or other segments (Chen et al., 2017; Feng et al., 2016; Zhong et al., 2018) to exhibit high virulence in chickens. Altogether, results from **Scheibner et al. (2019b)** are in line with the central role of the polybasic HACS in cleavability, wide dissemination into multiple organs and increased pathogenicity of AIV in chickens. Importantly, findings in this study showed that the polybasic HACS is the main determinant for endotheliotropism in chickens, but not in turkeys or ducks. Replication of the virus in the endothelial cells of chickens may result in coagulopathy, thrombosis, disruption of cell junctions and extravasation of erythrocytes and plasma resulting in cyanosis of comb and wattles, edema and/or death most likely due to shock (Pantin-Jackwood and Swayne, 2009). Moreover, the spread of HPAIV in chickens in different organs including the brain is mostly hematogenous in nature. This is also in accordance with results published by Kobayashi et al. (1996).

Turkeys are a common source for poultry meat worldwide (FAO, 2019a). They are known to be very susceptible to AIVs and generally show faster and more severe clinical signs than chickens (Halvorson et al., 1985; Karunakaran et al., 1983; Pantin-Jackwood and Swayne, 2009; Pillai et al., 2010). Particularly young turkeys are very sensitive to AIV infections with high mortality rates even after LPAIV infections as recorded in the H7N1 epidemic from 1999 in Italy (Capua et al., 2000a). Nevertheless, adult breeding turkeys show mostly clinical signs comparable to those observed in chickens (Lebarbenchon and Stallknecht, 2011). So far, most H7 subtypes such as H7N1, H7N2, H7N3, H7N6, H7N7, H7N8 and H7N9 have been found in turkeys (Abdelwhab el et al., 2013; Abdelwhab et al., 2014; Killian et al., 2016). The importance of the polybasic CS for virulence in turkeys was investigated in the first study **(Scheibner et al., 2019b)**. While clinical scoring of LP and HP H7N7 were comparable to those in chickens, LP-Poly was less virulent after oculonasal and intravenous inoculation. In turkeys results may indicate that genetic determinants for virulence are different from those in chickens. Since mutations were found in all eight gene segments in the current HPAIV H7N7 compared to the putative LP H7N7 precursor, the role of other gene segments in virulence in turkeys now has to be studied. It is worth mentioning that the only mutation which has already been investigated before is located in the NS1. The role of NS in virulence, transmission and/or replication of HPAIV *in vivo* and *in vitro* has been described (Abdelwhab et al., 2013; Li et al., 2006; Petersen et al., 2013). Importantly, HP H7N7 carries N92D, which is located in the NS1 effector domain.

It has already been found that mutation in position 92 (Glutamic acid or D) played a role in the interferon response in cell culture (Ngunjiri et al., 2013). However, the impact of NS1-N92D on virulence of HPAIV in turkeys has not been studied before. Other mutations may also modulate virulence in turkeys. V439A in the NA head domain may affect the NA activity and/or anti-NA antibody recognition (McAuley et al., 2019). The only other mutation within the HA is located in the signal peptide which may affect trafficking in the cytoplasm (Schafer et al., 1995). Given the pivotal role of M2 in uncoating, it is possible that the mutation M2-V68L, located in the cytoplasmic domain, may be necessary for pH activation of the virus (Cady et al., 2009). Moreover, since this mutation resides in the cytoplasmic domain of M2, an impact on virus budding through the interaction with M1 can not be excluded and will be further investigated.

Histopathological examination revealed limited antigen distribution in the intestinal tract and the pancreas for the LP virus, which is commonly observed in turkeys (Pantin-Jackwood and Swayne, 2009). In contrast, introduction of the polybasic CS led to a wide virus dissemination in different tissues similar to HP. The highest amounts of HP virus were found in the brain causing severe damage and severe neurological signs like seizures were remarkable. Interestingly, in contrast to chickens, nucleoprotein was not detected in the endothelium for any virus in turkeys. Narayan (1972) has shown that an H5N9 virus was able to spread to the brain causing severe damage without infection of endothelial cells. Similar results have been described in a more recent study where H5Nx inoculations lead to a wide host cell tropism without the infection of the endothelium (Carnaccini et al., 2019). On the other hand an HPAIV H7N8 from outbreaks in the USA in 2016 was detected in the vascular endothelium in experimentally infected turkeys (Pantin-Jackwood et al., 2017b). Three potential pathways of AIV neuroinvasion in birds have been described by Kobayashi et al. (1996): transport from the nasal cavity to the brain via the bulbus olfactorius, dissemination through the cardiovascular system, and infection of ependymal cells. While in chickens all three ways are conceivable, our findings suggest that the first and the last one are propably seen in turkeys. Endothelial infection in both species requires further investigation. Moreover, lymphatic depletion in the spleen and the lungs of turkeys was exclusively observed after HP infection indicating that mutations in other gene segments are important for inhibiting the host immune response (e.g. in spleen and lungs). Taken together, these findings reveal that the polybasic CS induces a wide tropism to several organs in turkeys. Unlike the hematogenous spread in chickens, systemic spread of HPAIV H7N7 in turkeys probably occurred via the nervous system, although the lymphatic pathway as a potential route for viral distribution is not excluded, since lymphatic organs were highly affected.

Ducks are known to be the natural reservoir of AIV. AIV infection in ducks is mostly asymptomatic with high virus replication and excretion (Alexander, 2000; Pantin-Jackwood and Swayne, 2009). Ducks are usually resistant to HP H5 or H7 infections and show only mild

clinical signs, if any, depending on the species and age of ducks as well as the challenge route (Kim et al., 2009; Pantin-Jackwood et al., 2017a). So far, only few HP infections in wild birds have been reported. The first was caused by an H5N3 in terns in 1961 in South Africa. Since 2002 H5N1 led to frequent infections of wild bird populations and the recent H5N8 from 2016 is also frequently detected in dead wild birds (Becker, 1966; Ellis et al., 2004; Ghafouri et al., 2019; Kishida et al., 2005; Muzyka et al., 2018; Pantin-Jackwood and Swayne, 2007; Soliman et al., 2012). Very little is known about H7 viruses in ducks (Kim et al., 2009; Pantin-Jackwood et al., 2017a; Pantin-Jackwood and Swayne, 2009). **Scheibner et al. (2019b)** described that infections of Pekin and Mallard ducks with the German HPAIV H7N7 were asymptomatic after ON or IV inoculation. Muscovy ducks were more sensitive, exhibited moderate clinical signs, and excreted higher virus levels which is in accordance with the higher susceptibility of Muscovy ducks to HPAIV H5Nx (Ellis et al., 2004; Kishida et al., 2005). **Scheibner et al. (2019a)** assessed the virulence of historic H7N1 and H7N7 from 1934 and 1927 outbreaks, respectively and a recent Italian H7N1 from 1999 epidemic in ducks. These viruses are highly virulent in chickens (Alexander et al., 1978; Capua et al., 2000a) but virulence in Muscovy ducklings was variable. While H7N1 from 1934 was highly virulent, H7N7 from 1927 and H7N1 from 1999 exhibited moderate virulence with a low mortality rate in Muscovy ducks after intrachoanal inoculation. These findings indicate that virulence determinants of HPAIV H7 in ducks differ from those in chickens. Viral and host factors that contribute to the virulence in chickens and (different species of) ducks remain to be investigated. Previous studies have shown that, in addition to a polybasic HACS, mutations in the polymerase genes conferred high virulence in ducks (Hulse-Post et al., 2007; Song et al., 2011). While ducks used for the experiments described by **Scheibner et al. (2019b)** were 4-weeks-old, the ducks used in the second study were 10-days-old **(Scheibner et al., 2019a)**. This difference in age may explain the lack of any mortality after inoculation with the German H7N7-2015 by **Scheibner et al. (2019b)**. Age dependent differences in susceptibility and virus transmission have already been reported for Mallard ducks (Costa et al., 2010). Therefore, it is important to assess the pathogenicity of this virus in young ducks. Together, experiments described in both studies included in this thesis showed that viral and host factors including virus strain, route of inoculation, species and probably age of ducks can modulate virulence of H7Nx viruses in ducks **(Scheibner et al., 2019a; Scheibner et al., 2019b)**.

In the study of **Scheibner et al. (2019b)**, Mallard (and Pekin) ducks showed no clinical signs after inoculation with the German H7N7. This is in accordance with the role of Mallard ducks as a main reservoir for AIV (Alexander, 2000; Munster et al., 2005; Pantin-Jackwood et al., 2017b; Pantin-Jackwood and Swayne, 2009). Moreover, Muscovy and Mallard ducks excreted considerable amounts of virus in the cloaca. This is in line with several studies describing high virus replication in the digestive tract of ducks leading to high cloacal shedding (Kida et al.,

1980; Slemons and Easterday, 1978; Webster et al., 1978). Conversely, all viruses tested in the study of **Scheibner et al. (2019a)** showed higher virus amounts in the oral swabs. Previous experiments demonstrated that some H7 viruses were also excreted mainly via the oral route (Pantin-Jackwood et al., 2016). Therefore, the impact of excretion of different HPAIV H7 via the oral and/or cloacal routes on virus spread (e.g. direct contact via oral route or contamination of water and feed via cloacal route) and the genetic determinants contributing to this altered excretion should be further investigated.

In conclusion, in this dissertation the virulence of H7 AIV in chickens, turkeys and ducks was investigated. While the polybasic CS led to almost full virulence and transmission in chickens, in turkeys other gene segments seem to be essential. Another striking difference is the endotheliotropism of HPAIV H7N7 in chickens, which was not observed in turkeys or ducks. In ducks, HPAIV H7 viruses exhibited variable virulence ranging from no signs to high mortality. Several factors including virus strain, duck species, route of inoculation and probably age at infection modulate virulence of HPAIV in ducks. In addition, Mallard ducks could play a crucial role for HP H7N7 spread among wild birds. These findings highlight variable molecular determinants and pathomechanisms for the evolution of HPAIV H7 in different bird species.

6 Summary

Avian influenza viruses of subtype H7Nx cause high economic losses and can evolve, similar to H5Nx viruses, to highly pathogenic viruses from low pathogenic ancestors. Some H7Nx viruses are endemic in Europe and are frequently reported to cause outbreaks in different poultry species. While turkeys and chickens are highly susceptible as indicated by high mortality, ducks are mostly clinically resistant to avian influenza virus (AIV).

In 2015 a natural pair of low pathogenic AIV (LPAIV)/ high pathogenic AIV (HPAIV) H7N7 virus was isolated from the same farm in the district Emsland, North West Germany. The genetic comparison between the LP and the HP H7N7 virus showed mutations in all eight segments and a polybasic cleavage site motif in the HP virus leading to high virulence and 100% mortality in chickens. In this thesis virulence determinants of this virus were investigated by reverse genetics and infection experiments in chickens, turkeys and ducks.

In the first publication we focused on the impact of the polybasic cleavage site of the H7N7 virus as a main virulence determinant in different bird species. While the polybasic cleavage site increased virulence and endothelial tropism of LPAIV in chickens to levels similar to the HP virus, it was less important but still necessary for high virulence in turkeys, but not in ducks. Pekin, Mallard and Muscovy ducks were susceptible to infection with HPAIV H7N7, but only Muscovy ducks showed mild to moderate clinical signs. Since Mallard ducks shed remarkable amounts of virus, they may play a role in the silent spread of HPAIV H7 viruses.

In the second publication, we assessed virulence of different European H7 viruses in Muscovy and Pekin ducks. In Muscovy ducks, the Fowl Plague Virus (FPV) H7N7 from 1927 caused 20% mortality, while the two H7N1 viruses from 1934 from Germany and from 1999 from Italy showed moderate to high virulence with a mortality rate ranging from 20 to 80% after intrachoanal inoculation. Although all viruses were highly virulent in chickens and possess a polybasic CS they exhibit variable virulence in ducks. These findings show the importance of the investigation of virulence determinants of H7 viruses in ducks.

Taken together, virulence determinants and pathobiology of HPAIV H7 is different according to the bird species. In chickens, the polybasic CS is the major determinant for virulence and tropism, while other gene segments are important for virulence and transmission in turkeys. Chickens and turkeys are dead-end hosts playing minimal role in the spread of HPAIV, whereas asymptomatically infected ducks can further spread the virus. These findings are crucial to understand the molecular biology of HPAIV in different bird species.

7 Zusammenfassung

Virulenz von aviären Influenzaviren des Subtypes H7 in Vögeln:

Der Einfluss der polybasischen Spaltstelle im Hämagglutinin auf die Virulenz eines aktuellen deutschen H7N7 Virus und die Pathogenität von europäischen H7N7 und H7N1 Viren in Enten

Aviäre Influenzviren (AIV) des Subtypes H7Nx verursachen große wirtschaftliche Schäden und können sich, genau wie H5Nx Viren, zu hoch virulenten Viren aus niedrig pathogenen Vorläufern entwickeln. Einige H7Nx Viren sind endemisch in Europa und führen regelmäßig zu Ausbrüchen in verschieden Geflügelspezies. Während Hühner und Puten sehr empfänglich sind, was durch eine hohe Mortalitätsrate gekennzeichnet ist, sind Enten meistens klinisch resistent gegen aviäre Influenzaviren.

2015 konnte ein natürliches Paar niedrig- (LP) und hochpathogener (HP) Phänotypen eines H7N7 Virus von einem Geflügelbetrieb im Landkreis Emsland, im Nordwesten Deutschlands, isoliert werden. Der genetische Vergleich beider Viren zeigte Mutationen in allen acht Segmenten und eine polybasische Spaltstelle im HP Virus, welche zu hoher Virulenz und 100% Mortalität in Hühnern führten. In dieser Arbeit wurden die Virulenzdeterminanten dieser Viren mithilfe reverser Genetik und Infektionsversuchen in Hühnern, Puten und Enten untersucht.

In der ersten Publikation lag das Augenmerk auf dem Einfluss der polybasischen Spaltstelle des H7N7 Viruses als Hauptvirulenzdeterminante in verschiedenen Vogelspezies. Während die polybasische Spaltstelle die Virulenz und den Endotheltropismus des niedrigpathogenen aviären Influenzaviruses (LPAIV) in Hühnern auf vergleichbare Werte wie das hochpathogene (HP) Virus erhöhte, war sie weniger wichtig, aber dennoch für hohe Virulenz in Puten notwendig, jedoch nicht in Enten. Peking-, Stock- und Moschusenten sind empfänglich für die Infektion mit HP H7N7, aber nur die Moschusenten zeigten ein mildes bis moderates Krankheitsbild. Da Stockenten beachtenswerte Mengen des Virus ausschieden, könnten sie eine Rolle als stille Überträger von hochpathogenen aviären Influenzaviren (HPAIVs) des Subtyps H7 spielen.

In der zweiten Publikation haben wir die Virulenz verschiedener europäischer H7 Viren in Moschus- und Pekingenten ermittelt. In Moschusenten verursachte das Fowl Plague Virus (FPV) H7N7 von 1927 eine Mortalität von 20%, während die beiden H7N1 Viren von 1934 aus Deutschland und von 1999 aus Italien moderate bis hohe Virulenz mit einer Mortalität von 20 bis 80% nach intrachoanaler Inokulation zeigten. Obwohl alle Viren hochvirulent in Hühnern waren und eine polybasische Spaltstelle aufwiesen, führten sie zu variabler Virulenz in Enten.

Diese Ergebnisse zeigen die Notwendigkeit der Untersuchung von Virulenzdeterminanten von H7 Viren in Enten.

Zusammenfassend ist zu sagen, dass die Virulenzdeterminanten und die Pathobiologie von HPAIV H7 in Bezug auf die Vogelspezies variiert. In Hühnern stellt die polybasische Spaltstelle die Hauptvirulenzdeterminante dar, während andere Gensegmente wichtig für die Virulenz und die Übertragung in Puten sind. Hühner und Puten sind Endwirte, die eine minimale Rolle für die Verbreitung von HPAIVs spielen, wobei asymptomatisch infizierte Enten diese Viren weiterverbreiten können. Diese Ergebnisse sind entscheidend für das Verständnis der Molekularbiologie von HPAIVs in verschiedenen Vogelspezies.

8 References (of Introduction and General discussion)

Aamir, U.B., K. Naeem, Z. Ahmed, C.A. Obert, J. Franks, S. Krauss, P. Seiler, and R.G. Webster. 2009. Zoonotic potential of highly pathogenic avian H7N3 influenza viruses from Pakistan. *Virology*. 390:212-220.

Abbas, M.A., E. Spackman, D.E. Swayne, Z. Ahmed, L. Sarmento, N. Siddique, K. Naeem, A. Hameed, and S. Rehmani. 2010. Sequence and phylogenetic analysis of H7N3 avian influenza viruses isolated from poultry in Pakistan 1995-2004. *Virol J*. 7:137.

Abdelwhab el, S.M., J. Veits, A. Breithaupt, S. Gohrbandt, M. Ziller, J.P. Teifke, J. Stech, and T.C. Mettenleiter. 2016. Prevalence of the C-terminal truncations of NS1 in avian influenza A viruses and effect on virulence and replication of a highly pathogenic H7N1 virus in chickens. *Virulence*. 7:546-557.

Abdelwhab el, S.M., J. Veits, and T.C. Mettenleiter. 2013. Genetic changes that accompanied shifts of low pathogenic avian influenza viruses toward higher pathogenicity in poultry. *Virulence*. 4:441-452.

Abdelwhab el, S.M., J. Veits, K. Tauscher, M. Ziller, J.P. Teifke, J. Stech, and T.C. Mettenleiter. 2015. A Unique Multibasic Proteolytic Cleavage Site and Three Mutations in the HA2 Domain Confer High Virulence of H7N1 Avian Influenza Virus in Chickens. *J Virol*. 90:400-411.

Abdelwhab, E.M., and H.M. Hafez. 2012. Insight into alternative approaches for control of avian influenza in poultry, with emphasis on highly pathogenic H5N1. *Viruses*. 4:3179-3208.

Abdelwhab, E.M., J. Veits, and T.C. Mettenleiter. 2013. Avian influenza virus NS1: A small protein with diverse and versatile functions. *Virulence*. 4:583-588.

Abdelwhab, E.M., J. Veits, and T.C. Mettenleiter. 2014. Prevalence and control of H7 avian influenza viruses in birds and humans. *Epidemiol Infect*. 142:896-920.

Abdelwhab, E.M., J. Veits, R. Ulrich, E. Kasbohm, J.P. Teifke, and T.C. Mettenleiter. 2016. Composition of the Hemagglutinin Polybasic Proteolytic Cleavage Motif Mediates Variable Virulence of H7N7 Avian Influenza Viruses. *Sci Rep*. 6:39505.

Abente, E.J., J. Santos, N.S. Lewis, P.C. Gauger, J. Stratton, E. Skepner, T.K. Anderson, D.S. Rajao, D.R. Perez, and A.L. Vincent. 2016. The Molecular Determinants of Antibody Recognition and Antigenic Drift in the H3 Hemagglutinin of Swine Influenza A Virus. *J Virol*. 90:8266-8280.

Ahlquist, P. 2002. RNA-dependent RNA polymerases, viruses, and RNA silencing. *Science*. 296:1270-1273.

Air, G.M. 2012. Influenza neuraminidase. *Influenza Other Respir Viruses*. 6:245-256.

Alexander, D.J. 2000. A review of avian influenza in different bird species. *Vet Microbiol*. 74:3-13.

Alexander, D.J. 2007. An overview of the epidemiology of avian influenza. *Vaccine*. 25:5637-5644.

Alexander, D.J., W.H. Allan, D.G. Parsons, and G. Parsons. 1978. The pathogenicity of four avian influenza viruses for fowls, turkeys and ducks. *Res Vet Sci*. 24:242-247.

Alexander, D.J., G. Parsons, and R.J. Manvell. 1986. Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtype for chickens, turkeys, ducks and quail. *Avian Pathol*. 15:647-662.

Alexander, D.J., and D. Spackman. 1981. Characterisation of influenza A viruses isolated from turkeys in England during March-May 1979. *Avian Pathol*. 10:281-293.

Allen, U.D., F.Y. Aoki, and H.G. Stiver. 2006. The use of antiviral drugs for influenza: recommended guidelines for practitioners. *Can J Infect Dis Med Microbiol*. 17:273-284.

Anthony, S.J., J.A. St Leger, K. Pugliares, H.S. Ip, J.M. Chan, Z.W. Carpenter, I. Navarrete-Macias, M. Sanchez-Leon, J.T. Saliki, J. Pedersen, W. Karesh, P. Daszak, R. Rabadan, T. Rowles, and W.I. Lipkin. 2012. Emergence of fatal avian influenza in New England harbor seals. *MBio*. 3:e00166-00112.

Arias, C.F., M. Escalera-Zamudio, L. Soto-Del Rio Mde, A.G. Cobian-Guemes, P. Isa, and S. Lopez. 2009. Molecular anatomy of 2009 influenza virus A (H1N1). *Arch Med Res*. 40:643-654.

Baigent, S.J., R.C. Bethell, and J.W. McCauley. 1999. Genetic analysis reveals that both haemagglutinin and neuraminidase determine the sensitivity of naturally occurring avian influenza viruses to zanamivir in vitro. *Virology*. 263:323-338.

Baigent, S.J., and J.W. McCauley. 2001. Glycosylation of haemagglutinin and stalk-length of neuraminidase combine to regulate the growth of avian influenza viruses in tissue culture. *Virus Res*. 79:177-185.

Banerjee, I., Y. Miyake, S.P. Nobs, C. Schneider, P. Horvath, M. Kopf, P. Matthias, A. Helenius, and Y. Yamauchi. 2014. Influenza A virus uses the aggresome processing machinery for host cell entry. *Science*. 346:473-477.

Banks, J., E.C. Speidel, J.W. McCauley, and D.J. Alexander. 2000. Phylogenetic analysis of H7 haemagglutinin subtype influenza A viruses. *Arch Virol*. 145:1047-1058.

Bano, S., K. Naeem, and S.A. Malik. 2003. Evaluation of pathogenic potential of avian influenza virus serotype H9N2 in chickens. *Avian Dis*. 47:817-822.

Barber, M.R., J.R. Aldridge, Jr., R.G. Webster, and K.E. Magor. 2010. Association of RIG-I with innate immunity of ducks to influenza. *Proc Natl Acad Sci U S A*. 107:5913-5918.

Barman, S., and D.P. Nayak. 2000. Analysis of the transmembrane domain of influenza virus neuraminidase, a type II transmembrane glycoprotein, for apical sorting and raft association. *J Virol*. 74:6538-6545.

Beard, C.W., and D.H. Helfer. 1972. Isolation of two turkey influenza viruses in Oregon. *Avian Dis*. 16:1133-1136.

Becker, W.B. 1966. The isolation and classification of Tern virus: influenza A-Tern South Africa--1961. *J Hyg (Lond)*. 64:309-320.

Belser, J.A., O. Blixt, L.M. Chen, C. Pappas, T.R. Maines, N. Van Hoeven, R. Donis, J. Busch, R. McBride, J.C. Paulson, J.M. Katz, and T.M. Tumpey. 2008. Contemporary North American influenza H7 viruses possess human receptor specificity: Implications for virus transmissibility. *Proc Natl Acad Sci U S A*. 105:7558-7563.

Belser, J.A., C.B. Bridges, J.M. Katz, and T.M. Tumpey. 2009a. Past, present, and possible future human infection with influenza virus A subtype H7. *Emerg Infect Dis*. 15:859-865.

Belser, J.A., N. Brock, X. Sun, J. Jones, N. Zanders, E. Hodges, J.A. Pulit-Penaloza, D. Wentworth, T.M. Tumpey, T. Davis, and T.R. Maines. 2018. Mammalian Pathogenesis and Transmission of Avian Influenza A(H7N9) Viruses, Tennessee, USA, 2017. *Emerg Infect Dis*. 24:149-152.

Belser, J.A., D.A. Wadford, J. Xu, J.M. Katz, and T.M. Tumpey. 2009b. Ocular infection of mice with influenza A (H7) viruses: a site of primary replication and spread to the respiratory tract. *J Virol*. 83:7075-7084.

Berhane, Y., T. Hisanaga, H. Kehler, J. Neufeld, L. Manning, C. Argue, K. Handel, K. Hooper-McGrevy, M. Jonas, J. Robinson, R.G. Webster, and J. Pasick. 2009. Highly pathogenic avian influenza virus A (H7N3) in domestic poultry, Saskatchewan, Canada, 2007. *Emerg Infect Dis*. 15:1492-1495.

Blok, J., and G.M. Air. 1982. Variation in the membrane-insertion and "stalk" sequences in eight subtypes of influenza type A virus neuraminidase. *Biochemistry*. 21:4001-4007.

Bodewes, R., S. Zohari, J.S. Krog, M.D. Hall, T.C. Harder, T.M. Bestebroer, M.W.G. van de Bildt, M.I. Spronken, L.E. Larsen, U. Siebert, P. Wohlsein, C. Puff, F. Seehusen, W. Baumgartner, T. Harkonen, S.L. Smits, S. Herfst, A. Osterhaus, R.A.M. Fouchier, M.P. Koopmans, and T. Kuiken. 2016. Spatiotemporal Analysis of the Genetic Diversity of Seal Influenza A(H10N7) Virus, Northwestern Europe. *J Virol*. 90:4269-4277.

Bogs, J., J. Veits, S. Gohrbandt, J. Hundt, O. Stech, A. Breithaupt, J.P. Teifke, T.C. Mettenleiter, and J. Stech. 2010. Highly pathogenic H5N1 influenza viruses carry virulence determinants beyond the polybasic hemagglutinin cleavage site. *PLoS One*. 5:e11826.

Boltz, D.A., J.R. Aldridge, Jr., R.G. Webster, and E.A. Govorkova. 2010. Drugs in development for influenza. *Drugs*. 70:1349-1362.

Bosch, F.X., W. Garten, H.D. Klenk, and R. Rott. 1981. Proteolytic Cleavage of Influenza-Virus Hemagglutinins - Primary Structure of the Connecting Peptide between Ha1 and Ha2

Determines Proteolytic Cleavability and Pathogenicity of Avian Influenza-Viruses. *Virology*. 113:725-735.

Bouvier, N.M., and P. Palese. 2008. The biology of influenza viruses. *Vaccine*. 26 Suppl 4:D49-53.

Bui, M., G. Whittaker, and A. Helenius. 1996. Effect of M1 protein and low pH on nuclear transport of influenza virus ribonucleoproteins. *J Virol*. 70:8391-8401.

Bullough, P.A., F.M. Hughson, J.J. Skehel, and D.C. Wiley. 1994. Structure of influenza haemagglutinin at the pH of membrane fusion. *Nature*. 371:37-43.

Byrd-Leotis, L., R.D. Cummings, and D.A. Steinhauer. 2017. The Interplay between the Host Receptor and Influenza Virus Hemagglutinin and Neuraminidase. *Int J Mol Sci*. 18.

Cady, S.D., W. Luo, F. Hu, and M. Hong. 2009. Structure and function of the influenza A M2 proton channel. *Biochemistry*. 48:7356-7364.

Calder, L.J., S. Wasilewski, J.A. Berriman, and P.B. Rosenthal. 2010. Structural organization of a filamentous influenza A virus. *Proc Natl Acad Sci U S A*. 107:10685-10690.

Callan, R.J., G. Early, H. Kida, and V.S. Hinshaw. 1995. The appearance of H3 influenza viruses in seals. *J Gen Virol*. 76 199-203.

Capua, I. 2008. Avian influenza in the EU. *Zoonoses Public Health*. 55:1.

Capua, I., and S. Marangon. 2007. The use of vaccination to combat multiple introductions of Notifiable Avian Influenza viruses of the H5 and H7 subtypes between 2000 and 2006 in Italy. *Vaccine*. 25:4987-4995.

Capua, I., S. Marangon, and F.M. Cancellotti. 2003. The 1999-2000 avian influenza (H7N1) epidemic in Italy. *Vet Res Commun*. 27 Suppl 1:123-127.

Capua, I., and F. Mutinelli. 2001. Mortality in Muscovy ducks (Cairina moschata) and domestic geese (Anser anser var. domestica) associated with natural infection with a highly pathogenic avian influenza virus of H7N1 subtype. *Avian Pathol*. 30:179-183.

Capua, I., F. Mutinelli, and M.H. Hablovarid. 2002. Avian embryo susceptibility to Italian H7N1 avian influenza viruses belonging to different genetic lineages. *Arch Virol*. 147:1611-1621.

Capua, I., F. Mutinelli, S. Marangon, and D.J. Alexander. 2000a. H7N1 avian influenza in Italy (1999 to 2000) in intensively reared chickens and turkeys. *Avian Pathol*. 29:537-543.

Capua, I., F. Mutinelli, C. Terregino, G. Cattoli, R.J. Manvell, and F. Burlini. 2000b. Highly pathogenic avian influenza (H7N1) in ostriches farmed in Italy. *Vet Rec*. 146:356.

Carnaccini, S., J.J.S. Santos, A.O. Obadan, M.J. Pantin-Jackwood, D.L. Suarez, D.S. Rajao, and D.R. Perez. 2019. Age-dependent pathogenesis of clade 2.3.4.4A H5N2 HPAIV in experimentally infected Broad Breasted White turkeys. *Vet Microbiol*. 231:183-190.

Chanturiya, A.N., G. Basanez, U. Schubert, P. Henklein, J.W. Yewdell, and J. Zimmerberg. 2004. PB1-F2, an influenza A virus-encoded proapoptotic mitochondrial protein, creates variably sized pores in planar lipid membranes. *J Virol*. 78:6304-6312.

Chare, E.R., E.A. Gould, and E.C. Holmes. 2003. Phylogenetic analysis reveals a low rate of homologous recombination in negative-sense RNA viruses. *J Gen Virol*. 84:2691-2703.

Chen, J., J.J. Skehel, and D.C. Wiley. 1999. N- and C-terminal residues combine in the fusion-pH influenza hemagglutinin HA(2) subunit to form an N cap that terminates the triple-stranded coiled coil. *Proc Natl Acad Sci U S A*. 96:8967-8972.

Chen, L., C. Wang, J. Luo, M. Li, H. Liu, N. Zhao, J. Huang, X. Zhu, G. Ma, G. Yuan, and H. He. 2017. Amino Acid Substitution K470R in the Nucleoprotein Increases the Virulence of H5N1 Influenza A Virus in Mammals. *Front Microbiol*. 8:1308.

Chen, R., and E.C. Holmes. 2006. Avian influenza virus exhibits rapid evolutionary dynamics. *Mol Biol Evol*. 23:2336-2341.

Chen, W., P.A. Calvo, D. Malide, J. Gibbs, U. Schubert, I. Bacik, S. Basta, R. O'Neill, J. Schickli, P. Palese, P. Henklein, J.R. Bennink, and J.W. Yewdell. 2001. A novel influenza A virus mitochondrial protein that induces cell death. *Nat Med*. 7:1306-1312.

Chen, Y., W. Liang, S. Yang, N. Wu, H. Gao, J. Sheng, H. Yao, J. Wo, Q. Fang, D. Cui, Y. Li, X. Yao, Y. Zhang, H. Wu, S. Zheng, H. Diao, S. Xia, Y. Zhang, K.H. Chan, H.W. Tsoi, J.L. Teng, W. Song, P. Wang, S.Y. Lau, M. Zheng, J.F. Chan, K.K. To, H. Chen, L. Li, and K.Y. Yuen. 2013. Human infections with the emerging avian influenza A H7N9 virus from wet market poultry: clinical analysis and characterisation of viral genome. *Lancet*. 381:1916-1925.

Colman, P.M. 1994. Influenza virus neuraminidase: structure, antibodies, and inhibitors. *Protein Sci*. 3:1687-1696.

Connor, R.J., Y. Kawaoka, R.G. Webster, and J.C. Paulson. 1994. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. *Virology*. 205:17-23.

Costa, T.P., J.D. Brown, E.W. Howerth, and D.E. Stallknecht. 2010. The effect of age on avian influenza viral shedding in mallards (Anas platyrhynchos). *Avian Dis*. 54:581-585.

De Conto, F., S. Covan, M.C. Arcangeletti, G. Orlandini, R. Gatti, G. Dettori, and C. Chezzi. 2011. Differential infectious entry of human influenza A/NWS/33 virus (H1N1) in mammalian kidney cells. *Virus Res*. 155:221-230.

de Graaf, M., and R.A. Fouchier. 2014. Role of receptor binding specificity in influenza A virus transmission and pathogenesis. *EMBO J*. 33:823-841.

de Jong, M.C., A. Stegeman, J. van der Goot, and G. Koch. 2009. Intra- and interspecies transmission of H7N7 highly pathogenic avian influenza virus during the avian influenza epidemic in The Netherlands in 2003. *Revue scientifique et technique*. 28:333-340.

de Vries, R.D., and G.F. Rimmelzwaan. 2016. Viral vector-based influenza vaccines. *Human vaccines & immunotherapeutics*. 12:2881-2901.

de Wit, J.J., G. Koch, T.H. Fabri, and A.R. Elbers. 2004. A cross-sectional serological survey of the Dutch commercial poultry population for the presence of low pathogenic avian influenza virus infections. *Avian Pathol*. 33:565-570.

DeLay, P.D., H.L. Casey, and H.S. Tubiash. 1967. Comparative study of fowl plague virus and a virus isolated from man. *Public Health Rep*. 82:615-620.

Deshpande, K.L., V.A. Fried, M. Ando, and R.G. Webster. 1987. Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence. *Proc Natl Acad Sci U S A*. 84:36-40.

Dias, A., D. Bouvier, T. Crepin, A.A. McCarthy, D.J. Hart, F. Baudin, S. Cusack, and R.W. Ruigrok. 2009. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. *Nature*. 458:914-918.

Dietze, K., A. Graaf, T. Homeier-Bachmann, C. Grund, L. Forth, A. Pohlmann, C. Jeske, M. Wintermann, M. Beer, F.J. Conraths, and T. Harder. 2018. From low to high pathogenicity-Characterization of H7N7 avian influenza viruses in two epidemiologically linked outbreaks. *Transboundary and emerging diseases*. 65:1576-1587.

Dong, G., C. Peng, J. Luo, C. Wang, L. Han, B. Wu, G. Ji, and H. He. 2015. Adamantane-resistant influenza a viruses in the world (1902-2013): frequency and distribution of M2 gene mutations. *PLoS One*. 10:e0119115.

Dortmans, J.C., J. Dekkers, I.N. Wickramasinghe, M.H. Verheije, P.J. Rottier, F.J. van Kuppeveld, E. de Vries, and C.A. de Haan. 2013. Adaptation of novel H7N9 influenza A virus to human receptors. *Sci Rep*. 3:3058.

Du, J., M.A. Meledeo, Z. Wang, H.S. Khanna, V.D. Paruchuri, and K.J. Yarema. 2009. Metabolic glycoengineering: sialic acid and beyond. *Glycobiology*. 19:1382-1401.

Durrant, J.D., R.M. Bush, and R.E. Amaro. 2016. Microsecond Molecular Dynamics Simulations of Influenza Neuraminidase Suggest a Mechanism for the Increased Virulence of Stalk-Deletion Mutants. *J Phys Chem B*. 120:8590-8599.

Durrer, P., C. Galli, S. Hoenke, C. Corti, R. Gluck, T. Vorherr, and J. Brunner. 1996. H+-induced membrane insertion of influenza virus hemagglutinin involves the HA2 amino-terminal fusion peptide but not the coiled coil region. *J Biol Chem*. 271:13417-13421.

Eisfeld, A.J., G. Neumann, and Y. Kawaoka. 2015. At the centre: influenza A virus ribonucleoproteins. *Nat Rev Microbiol*. 13:28-41.

Ellis, T.M., R.B. Bousfield, L.A. Bissett, K.C. Dyrting, G.S. Luk, S.T. Tsim, K. Sturm-Ramirez, R.G. Webster, Y. Guan, and J.S. Malik Peiris. 2004. Investigation of outbreaks of highly

pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. *Avian Pathol*. 33:492-505.

Enami, M., and K. Enami. 1996. Influenza virus hemagglutinin and neuraminidase glycoproteins stimulate the membrane association of the matrix protein. *J Virol*. 70:6653-6657.

Engelhardt, O.G., M. Smith, and E. Fodor. 2005. Association of the influenza A virus RNA-dependent RNA polymerase with cellular RNA polymerase II. *J Virol*. 79:5812-5818.

Englund, L., B. Klingeborn, and T. Mejerland. 1986. Avian influenza A virus causing an outbreak of contagious interstitial pneumonia in mink. *Acta Vet Scand*. 27:497-504.

FAO. 2019a. Gateway to poultry production and products http://www.fao.org/poultry-production-products/production/poultry-species/en/ last accessed on 20.06.19.

FAO. 2019b. H7N9 situation update, http://www.fao.org/ag/againfo/programmes/en/empres/H7N9/situation_update.html , update from 3.07.19, last accessed on 28.07.19.

Feng, X., Z. Wang, J. Shi, G. Deng, H. Kong, S. Tao, C. Li, L. Liu, Y. Guan, and H. Chen. 2016. Glycine at Position 622 in PB1 Contributes to the Virulence of H5N1 Avian Influenza Virus in Mice. *J Virol*. 90:1872-1879.

Ferguson, N.M., A.P. Galvani, and R.M. Bush. 2003. Ecological and immunological determinants of influenza evolution. *Nature*. 422:428-433.

Fontana, J., G. Cardone, J.B. Heymann, D.C. Winkler, and A.C. Steven. 2012. Structural changes in Influenza virus at low pH characterized by cryo-electron tomography. *J Virol*. 86:2919-2929.

Fouchier, R.A., V. Munster, A. Wallensten, T.M. Bestebroer, S. Herfst, D. Smith, G.F. Rimmelzwaan, B. Olsen, and A.D. Osterhaus. 2005. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. *J Virol*. 79:2814-2822.

Fouchier, R.A., P.M. Schneeberger, F.W. Rozendaal, J.M. Broekman, S.A. Kemink, V. Munster, T. Kuiken, G.F. Rimmelzwaan, M. Schutten, G.J. Van Doornum, G. Koch, A. Bosman, M. Koopmans, and A.D. Osterhaus. 2004. Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. *Proc Natl Acad Sci U S A*. 101:1356-1361.

Fournier, E., V. Moules, B. Essere, J.C. Paillart, J.D. Sirbat, C. Isel, A. Cavalier, J.P. Rolland, D. Thomas, B. Lina, and R. Marquet. 2012. A supramolecular assembly formed by influenza A virus genomic RNA segments. *Nucleic Acids Res*. 40:2197-2209.

Franca, M., D.E. Stallknecht, R. Poulson, J. Brown, and E.W. Howerth. 2012. The pathogenesis of low pathogenic avian influenza in mallards. *Avian Dis*. 56:976-980.

Fujii, Y., H. Goto, T. Watanabe, T. Yoshida, and Y. Kawaoka. 2003. Selective incorporation of influenza virus RNA segments into virions. *Proc Natl Acad Sci U S A*. 100:2002-2007.

Fujimoto, Y., H. Ito, M. Tomita, E. Ono, T. Usui, and T. Ito. 2015. Amino acid substitution at position 44 of matrix protein 2 of an avirulent H5 avian influenza virus is crucial for acquiring the highly pathogenic phenotype in chickens. *Arch Virol*. 160:2063-2070.

Gabriel, G., B. Dauber, T. Wolff, O. Planz, H.D. Klenk, and J. Stech. 2005. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. *Proc Natl Acad Sci U S A*. 102:18590-18595.

Gamblin, S.J., L.F. Haire, R.J. Russell, D.J. Stevens, B. Xiao, Y. Ha, N. Vasisht, D.A. Steinhauer, R.S. Daniels, A. Elliot, D.C. Wiley, and J.J. Skehel. 2004. The structure and receptor binding properties of the 1918 influenza hemagglutinin. *Science*. 303:1838-1842.

Gamblin, S.J., and J.J. Skehel. 2010. Influenza hemagglutinin and neuraminidase membrane glycoproteins. *J Biol Chem*. 285:28403-28409.

Ghafouri, S.A., M.H. Fallah Mehrabadi, S.F. Talakesh, H. Hosseini, Z. Ziafati, M. Malekan, L. Aghaeean, and A. Ghalyanchilangeroudi. 2019. Full genome characterization of Iranian H5N8 highly pathogenic avian influenza virus from Hooded Crow (Corvus cornix), 2017: The first report. *Comp Immunol Microbiol Infect Dis*. 64:73-80.

Ghedin, E., N.A. Sengamalay, M. Shumway, J. Zaborsky, T. Feldblyum, V. Subbu, D.J. Spiro, J. Sitz, H. Koo, P. Bolotov, D. Dernovoy, T. Tatusova, Y. Bao, K. St George, J. Taylor, D.J. Lipman, C.M. Fraser, J.K. Taubenberger, and S.L. Salzberg. 2005. Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. *Nature*. 437:1162-1166.

Grund, C., D. Hoffmann, R. Ulrich, M. Naguib, J. Schinkothe, B. Hoffmann, T. Harder, S. Saenger, K. Zscheppang, M. Tonnies, S. Hippenstiel, A. Hocke, T. Wolff, and M. Beer. 2018. A novel European H5N8 influenza A virus has increased virulence in ducks but low zoonotic potential. *Emerg Microbes Infect*. 7:132.

Guo, Y., M. Wang, Y. Kawaoka, O. Gorman, T. Ito, T. Saito, and R.G. Webster. 1992. Characterization of a new avian-like influenza A virus from horses in China. *Virology*. 188:245-255.

Guo, Y., M. Wang, G.S. Zheng, W.K. Li, Y. Kawaoka, and R.G. Webster. 1995. Seroepidemiological and molecular evidence for the presence of two H3N8 equine influenza viruses in China in 1993-94. *J Gen Virol*. 76:2009-2014.

Halvorson, D.A., C.J. Kelleher, and D.A. Senne. 1985. Epizootiology of avian influenza: effect of season on incidence in sentinel ducks and domestic turkeys in Minnesota. *Applied and environmental microbiology*. 49:914-919.

Hinshaw, V.S., W.J. Bean, J. Geraci, P. Fiorelli, G. Early, and R.G. Webster. 1986. Characterization of two influenza A viruses from a pilot whale. *J Virol*. 58:655-656.

Hinshaw, V.S., W.J. Bean, R.G. Webster, J.E. Rehg, P. Fiorelli, G. Early, J.R. Geraci, and D.J. St Aubin. 1984. Are seals frequently infected with avian influenza viruses? *J Virol*. 51:863-865.

Hinshaw, V.S., R.G. Webster, and B. Turner. 1979. Water-bone transmission of influenza A viruses? *Intervirology*. 11:66-68.

Hinshaw, V.S., R.G. Webster, and B. Turner. 1980. The perpetuation of orthomyxoviruses and paramyxoviruses in Canadian waterfowl. *Can J Microbiol*. 26:622-629.

Holsinger, L.J., and R.A. Lamb. 1991. Influenza virus M2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. *Virology*. 183:32-43.

Hu, J., Z. Hu, Y. Mo, Q. Wu, Z. Cui, Z. Duan, J. Huang, H. Chen, Y. Chen, M. Gu, X. Wang, S. Hu, H. Liu, W. Liu, X. Liu, and X. Liu. 2013. The PA and HA gene-mediated high viral load and intense innate immune response in the brain contribute to the high pathogenicity of H5N1 avian influenza virus in mallard ducks. *J Virol*. 87:11063-11075.

Hu, J., Y. Mo, X. Wang, M. Gu, Z. Hu, L. Zhong, Q. Wu, X. Hao, S. Hu, W. Liu, H. Liu, X. Liu, and X. Liu. 2015. PA-X decreases the pathogenicity of highly pathogenic H5N1 influenza A virus in avian species by inhibiting virus replication and host response. *J Virol*. 89:4126-4142.

Hu, Y., H. Sneyd, R. Dekant, and J. Wang. 2017. Influenza A Virus Nucleoprotein: A Highly Conserved Multi-Functional Viral Protein as a Hot Antiviral Drug Target. *Curr Top Med Chem*. 17:2271-2285.

Hulse-Post, D.J., J. Franks, K. Boyd, R. Salomon, E. Hoffmann, H.L. Yen, R.J. Webby, D. Walker, T.D. Nguyen, and R.G. Webster. 2007. Molecular changes in the polymerase genes (PA and PB1) associated with high pathogenicity of H5N1 influenza virus in mallard ducks. *J Virol*. 81:8515-8524.

Hurt, A.C., T. Chotpitayasunondh, N.J. Cox, R. Daniels, A.M. Fry, L.V. Gubareva, F.G. Hayden, D.S. Hui, O. Hungnes, A. Lackenby, W. Lim, A. Meijer, C. Penn, M. Tashiro, T.M. Uyeki, M. Zambon, and W.H.O.C.o.P.I.A.V.R.t. Antivirals. 2012. Antiviral resistance during the 2009 influenza A H1N1 pandemic: public health, laboratory, and clinical perspectives. *Lancet Infect Dis*. 12:240-248.

Hussain, M., H.D. Galvin, T.Y. Haw, A.N. Nutsford, and M. Husain. 2017. Drug resistance in influenza A virus: the epidemiology and management. *Infect Drug Resist*. 10:121-134.

Hutchinson, E.C., E.M. Denham, B. Thomas, D.C. Trudgian, S.S. Hester, G. Ridlova, A. York, L. Turrell, and E. Fodor. 2012. Mapping the phosphoproteome of influenza A and B viruses by mass spectrometry. *PLoS Pathog*. 8:e1002993.

Hutchinson, E.C., J.C. von Kirchbach, J.R. Gog, and P. Digard. 2010. Genome packaging in influenza A virus. *J Gen Virol*. 91:313-328.

Isin, B., P. Doruker, and I. Bahar. 2002. Functional motions of influenza virus hemagglutinin: a structure-based analytical approach. *Biophys J*. 82:569-581.

Jagger, B.W., H.M. Wise, J.C. Kash, K.A. Walters, N.M. Wills, Y.L. Xiao, R.L. Dunfee, L.M. Schwartzman, A. Ozinsky, G.L. Bell, R.M. Dalton, A. Lo, S. Efstathiou, J.F. Atkins, A.E. Firth, J.K. Taubenberger, and P. Digard. 2012. An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. *Science*. 337:199-204.

Jeong, O.M., M.C. Kim, M.J. Kim, H.M. Kang, H.R. Kim, Y.J. Kim, S.J. Joh, J.H. Kwon, and Y.J. Lee. 2009. Experimental infection of chickens, ducks and quails with the highly pathogenic H5N1 avian influenza virus. *J Vet Sci*. 10:53-60.

Jourdain, E., G. Gunnarsson, J. Wahlgren, N. Latorre-Margalef, C. Brojer, S. Sahlin, L. Svensson, J. Waldenstrom, A. Lundkvist, and B. Olsen. 2010. Influenza virus in a natural host, the mallard: experimental infection data. *PLoS One*. 5:e8935.

Kalthoff, D., A. Globig, and M. Beer. 2010. (Highly pathogenic) avian influenza as a zoonotic agent. *Vet Microbiol*. 140:237-245.

Kapczynski, D.R., M. Pantin-Jackwood, S.G. Guzman, Y. Ricardez, E. Spackman, K. Bertran, D.L. Suarez, and D.E. Swayne. 2013. Characterization of the 2012 highly pathogenic avian influenza H7N3 virus isolated from poultry in an outbreak in Mexico: pathobiology and vaccine protection. *J Virol*. 87:9086-9096.

Karlsson, E.A., H.S. Ip, J.S. Hall, S.W. Yoon, J. Johnson, M.A. Beck, R.J. Webby, and S. Schultz-Cherry. 2014. Respiratory transmission of an avian H3N8 influenza virus isolated from a harbour seal. *Nat Commun*. 5:4791.

Karunakaran, D., V. Hinshaw, P. Poss, J. Newman, and D. Halvorson. 1983. Influenza A outbreaks in Minnesota turkeys due to subtype H10N7 and possible transmission by waterfowl. *Avian Dis*. 27:357-366.

Kato, N., and H.J. Eggers. 1969. Inhibition of uncoating of fowl plague virus by l-adamantanamine hydrochloride. *Virology*. 37:632-641.

Kawaoka, Y., T.M. Chambers, W.L. Sladen, and R.G. Webster. 1988. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? *Virology*. 163:247-250.

Kawaoka, Y., and R.G. Webster. 1989. Interplay between carbohydrate in the stalk and the length of the connecting peptide determines the cleavability of influenza virus hemagglutinin. *J Virol*. 63:3296-3300.

Kemble, G.W., D.L. Bodian, J. Rose, I.A. Wilson, and J.M. White. 1992. Intermonomer disulfide bonds impair the fusion activity of influenza virus hemagglutinin. *J Virol*. 66:4940-4950.

Kida, H., R. Yanagawa, and Y. Matsuoka. 1980. Duck influenza lacking evidence of disease signs and immune response. *Infect Immun*. 30:547-553.

Killian, M.L., M. Kim-Torchetti, N. Hines, S. Yingst, T. DeLiberto, and D.H. Lee. 2016. Outbreak of H7N8 Low Pathogenic Avian Influenza in Commercial Turkeys with Spontaneous Mutation to Highly Pathogenic Avian Influenza. *Genome Announc*. 4.

Kim, J.K., N.J. Negovetich, H.L. Forrest, and R.G. Webster. 2009. Ducks: the "Trojan horses" of H5N1 influenza. *Influenza Other Respir Viruses*. 3:121-128.

Kishida, N., Y. Sakoda, N. Isoda, K. Matsuda, M. Eto, Y. Sunaga, T. Umemura, and H. Kida. 2005. Pathogenicity of H5 influenza viruses for ducks. *Arch Virol*. 150:1383-1392.

Klenk, H.D., and W. Garten. 1994. Host cell proteases controlling virus pathogenicity. *Trends Microbiol*. 2:39-43.

Klingeborn, B., L. Englund, R. Rott, N. Juntti, and G. Rockborn. 1985. An avian influenza A virus killing a mammalian species--the mink. Brief report. *Arch Virol*. 86:347-351.

Kobayashi, Y., T. Horimoto, Y. Kawaoka, D.J. Alexander, and C. Itakura. 1996. Neuropathological studies of chickens infected with highly pathogenic avian influenza viruses. *J Comp Pathol*. 114:131-147.

Koopmans, M., B. Wilbrink, M. Conyn, G. Natrop, H. van der Nat, H. Vennema, A. Meijer, J. van Steenbergen, R. Fouchier, A. Osterhaus, and A. Bosman. 2004. Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. *Lancet*. 363:587-593.

Kuchipudi, S.V., M. Tellabati, S. Sebastian, B.Z. Londt, C. Jansen, L. Vervelde, S.M. Brookes, I.H. Brown, S.P. Dunham, and K.C. Chang. 2014. Highly pathogenic avian influenza virus infection in chickens but not ducks is associated with elevated host immune and pro-inflammatory responses. *Vet Res*. 45:118.

Kuiken, T., G. Rimmelzwaan, D. van Riel, G. van Amerongen, M. Baars, R. Fouchier, and A. Osterhaus. 2004. Avian H5N1 influenza in cats. *Science*. 306:241.

Kung, N.Y., Y. Guan, N.R. Perkins, L. Bissett, T. Ellis, L. Sims, R.S. Morris, K.F. Shortridge, and J.S. Peiris. 2003. The impact of a monthly rest day on avian influenza virus isolation rates in retail live poultry markets in Hong Kong. *Avian Dis*. 47:1037-1041.

Lakadamyali, M., M.J. Rust, H.P. Babcock, and X. Zhuang. 2003. Visualizing infection of individual influenza viruses. *Proc Natl Acad Sci U S A*. 100:9280-9285.

Lam, T.T., J. Wang, Y. Shen, B. Zhou, L. Duan, C.L. Cheung, C. Ma, S.J. Lycett, C.Y. Leung, X. Chen, L. Li, W. Hong, Y. Chai, L. Zhou, H. Liang, Z. Ou, Y. Liu, A. Farooqui, D.J. Kelvin, L.L. Poon, D.K. Smith, O.G. Pybus, G.M. Leung, Y. Shu, R.G. Webster, R.J. Webby, J.S. Peiris, A. Rambaut, H. Zhu, and Y. Guan. 2013. The genesis and source of the H7N9 influenza viruses causing human infections in China. *Nature*. 502:241-244.

Lang, G., A. Gagnon, and J.R. Geraci. 1981. Isolation of an influenza A virus from seals. *Arch Virol*. 68:189-195.

Laver, W.G. 1971. Separation of two polypeptide chains from the hemagglutinin subunit of influenza virus. *Virology*. 45:275-288.

Lebarbenchon, C., and D.E. Stallknecht. 2011. Host shifts and molecular evolution of H7 avian influenza virus hemagglutinin. *Virol J*. 8:328.

Lee, C.W., D.L. Suarez, T.M. Tumpey, H.W. Sung, Y.K. Kwon, Y.J. Lee, J.G. Choi, S.J. Joh, M.C. Kim, E.K. Lee, J.M. Park, X. Lu, J.M. Katz, E. Spackman, D.E. Swayne, and J.H. Kim. 2005. Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. *J Virol*. 79:3692-3702.

Lee, D.H., M.K. Torchetti, M.L. Killian, Y. Berhane, and D.E. Swayne. 2017. Highly Pathogenic Avian Influenza A(H7N9) Virus, Tennessee, USA, March 2017. *Emerg Infect Dis*. 23.

Li, J., H. Zu Dohna, C.J. Cardona, J. Miller, and T.E. Carpenter. 2011. Emergence and genetic variation of neuraminidase stalk deletions in avian influenza viruses. *PLoS One*. 6:e14722.

Li, K.S., K.M. Xu, J.S. Peiris, L.L. Poon, K.Z. Yu, K.Y. Yuen, K.F. Shortridge, R.G. Webster, and Y. Guan. 2003. Characterization of H9 subtype influenza viruses from the ducks of southern China: a candidate for the next influenza pandemic in humans? *J Virol*. 77:6988-6994.

Li, Z., Y. Jiang, P. Jiao, A. Wang, F. Zhao, G. Tian, X. Wang, K. Yu, Z. Bu, and H. Chen. 2006. The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. *J Virol*. 80:11115-11123.

Londt, B.Z., J. Banks, and D.J. Alexander. 2007. Highly pathogenic avian influenza viruses with low virulence for chickens in in vivo tests. *Avian Pathol*. 36:347-350.

Lukarska, M., G. Fournier, A. Pflug, P. Resa-Infante, S. Reich, N. Naffakh, and S. Cusack. 2017. Structural basis of an essential interaction between influenza polymerase and Pol II CTD. *Nature*. 541:117-121.

Lupiani, B., and S.M. Reddy. 2009. The history of avian influenza. *Comp Immunol Microbiol Infect Dis*. 32:311-323.

Magnino, S., M. Fabbi, A. Moreno, G. Sala, A. Lavazza, E. Ghelfi, L. Gandolfi, G. Pirovano, and E. Gasperi. 2000. Avian influenza virus (H7 serotype) in a saker falcon in Italy. *Vet Rec*. 146:740.

Magor, K.E., D. Miranzo Navarro, M.R. Barber, K. Petkau, X. Fleming-Canepa, G.A. Blyth, and A.H. Blaine. 2013. Defense genes missing from the flight division. *Dev Comp Immunol*. 41:377-388.

Marc, D. 2014. Influenza virus non-structural protein NS1: interferon antagonism and beyond. *J Gen Virol*. 95:2594-2611.

Matsuoka, Y., D.E. Swayne, C. Thomas, M.A. Rameix-Welti, N. Naffakh, C. Warnes, M. Altholtz, R. Donis, and K. Subbarao. 2009. Neuraminidase stalk length and additional glycosylation of the hemagglutinin influence the virulence of influenza H5N1 viruses for mice. *J Virol*. 83:4704-4708.

Maurer-Stroh, S., R.T. Lee, V. Gunalan, and F. Eisenhaber. 2013. The highly pathogenic H7N3 avian influenza strain from July 2012 in Mexico acquired an extended cleavage site through recombination with host 28S rRNA. *Virol J*. 10:139.

Max, V., J. Herrera, R. Moreira, and H. Rojas. 2007. Avian influenza in Chile: a successful experience. *Avian Dis*. 51:363-365.

McAuley, J.L., B.P. Gilbertson, S. Trifkovic, L.E. Brown, and J.L. McKimm-Breschkin. 2019. Influenza Virus Neuraminidase Structure and Functions. *Front Microbiol*. 10:39.

McKimm-Breschkin, J.L. 2013. Influenza neuraminidase inhibitors: antiviral action and mechanisms of resistance. *Influenza Other Respir Viruses*. 7 Suppl 1:25-36.

Meijer, A., A. Bosman, E.E. van de Kamp, B. Wilbrink, M. Du Ry van Beest Holle, and M. Koopmans. 2006. Measurement of antibodies to avian influenza virus A(H7N7) in humans by hemagglutination inhibition test. *J Virol Methods*. 132:113-120.

Morens, D.M., and J.K. Taubenberger. 2011. Pandemic influenza: certain uncertainties. *Rev Med Virol*. 21:262-284.

Morens, D.M., J.K. Taubenberger, G.K. Folkers, and A.S. Fauci. 2010. Pandemic influenza's 500th anniversary. *Clin Infect Dis*. 51:1442-1444.

Mostafa, A., S.M. Abdelwhab el, H. Slanina, M.A. Hussein, I. Kuznetsova, C.G. Schuttler, J. Ziebuhr, and S. Pleschka. 2016. Phylogenetic analysis of human influenza A/H3N2 viruses isolated in 2015 in Germany indicates significant genetic divergence from vaccine strains. *Arch Virol*. 161:1505-1515.

Mostafa, A., E.M. Abdelwhab, T.C. Mettenleiter, and S. Pleschka. 2018. Zoonotic Potential of Influenza A Viruses: A Comprehensive Overview. *Viruses*. 10.

Munoz, E.T., and M.W. Deem. 2005. Epitope analysis for influenza vaccine design. *Vaccine*. 23:1144-1148.

Munster, V.J., E.J. Schrauwen, E. de Wit, J.M. van den Brand, T.M. Bestebroer, S. Herfst, G.F. Rimmelzwaan, A.D. Osterhaus, and R.A. Fouchier. 2010. Insertion of a multibasic cleavage motif into the hemagglutinin of a low-pathogenic avian influenza H6N1 virus induces a highly pathogenic phenotype. *J Virol*. 84:7953-7960.

Munster, V.J., A. Wallensten, C. Baas, G.F. Rimmelzwaan, M. Schutten, B. Olsen, A.D. Osterhaus, and R.A. Fouchier. 2005. Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe. *Emerg Infect Dis*. 11:1545-1551.

Muramoto, Y., T. Noda, E. Kawakami, R. Akkina, and Y. Kawaoka. 2013. Identification of novel influenza A virus proteins translated from PA mRNA. *J Virol*. 87:2455-2462.

Muzyka, D., O. Rula, S. Tkachenko, N. Muzyka, S. Kothe, O. Pishchanskyi, B. Stegniy, M. Pantin-Jackwood, and M. Beer. 2018. Highly Pathogenic and Low Pathogenic Avian Influenza H5 Subtype Viruses in Wild Birds in Ukraine. *Avian Dis*. 63:219-229.

Naeem, K., and M. Hussain. 1995. An outbreak of avian influenza in poultry in Pakistan. *Vet Rec*. 137:439.

Naeem, K., N. Siddique, M. Ayaz, and M.A. Jalalee. 2007. Avian influenza in Pakistan: outbreaks of low- and high-pathogenicity avian influenza in Pakistan during 2003-2006. *Avian Dis*. 51:189-193.

Naeve, C.W., and D. Williams. 1990. Fatty acids on the A/Japan/305/57 influenza virus hemagglutinin have a role in membrane fusion. *EMBO J*. 9:3857-3866.

Nagy, A., T.C. Mettenleiter, and E.M. Abdelwhab. 2017. A brief summary of the epidemiology and genetic relatedness of avian influenza H9N2 virus in birds and mammals in the Middle East and North Africa. *Epidemiol Infect*. 145:3320-3333.

Nao, N., M. Kajihara, R. Manzoor, J. Maruyama, R. Yoshida, M. Muramatsu, H. Miyamoto, M. Igarashi, N. Eguchi, M. Sato, T. Kondoh, M. Okamatsu, Y. Sakoda, H. Kida, and A. Takada. 2015. A Single Amino Acid in the M1 Protein Responsible for the Different Pathogenic Potentials of H5N1 Highly Pathogenic Avian Influenza Virus Strains. *PLoS One*. 10:e0137989.

Narayan, O. 1972. Pathogenesis of lethal influenza virus infection in turkeys. II. Central nervous system phase of infection. *J Comp Pathol*. 82:139-146.

Ng, A.K., H. Zhang, K. Tan, Z. Li, J.H. Liu, P.K. Chan, S.M. Li, W.Y. Chan, S.W. Au, A. Joachimiak, T. Walz, J.H. Wang, and P.C. Shaw. 2008. Structure of the influenza virus A H5N1 nucleoprotein: implications for RNA binding, oligomerization, and vaccine design. *FASEB J*. 22:3638-3647.

Ngunjiri, J.M., G.M. Buchek, K.N. Mohni, M.J. Sekellick, and P.I. Marcus. 2013. Influenza virus subpopulations: exchange of lethal H5N1 virus NS for H1N1 virus NS triggers de novo generation of defective-interfering particles and enhances interferon-inducing particle efficiency. *J Interferon Cytokine Res*. 33:99-107.

Nguyen-Van-Tam, J.S., P. Nair, P. Acheson, A. Baker, M. Barker, S. Bracebridge, J. Croft, J. Ellis, R. Gelletlie, N. Gent, S. Ibbotson, C. Joseph, H. Mahgoub, P. Monk, T.W. Reghitt, T. Sundkvist, C. Sellwood, J. Simpson, J. Smith, J.M. Watson, M. Zambon, N. Lightfoot, and T. Incident Response. 2006. Outbreak of low pathogenicity H7N3 avian influenza in UK, including associated case of human conjunctivitis. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin*. 11:E060504 060502.

Nguyen, H.T., T. Nguyen, V.P. Mishin, K. Sleeman, A. Balish, J. Jones, A. Creanga, H. Marjuki, T.M. Uyeki, D.H. Nguyen, D.T. Nguyen, H.T. Do, A.I. Klimov, C.T. Davis, and L.V.

Gubareva. 2013. Antiviral susceptibility of highly pathogenic avian influenza A(H5N1) viruses isolated from poultry, Vietnam, 2009-2011. *Emerg Infect Dis*. 19:1963-1971.

Nielsen, O., A. Clavijo, and J.A. Boughen. 2001. Serologic evidence of influenza A infection in marine mammals of arctic Canada. *J Wildl Dis*. 37:820-825.

Nilsson, B.E., A.J.W. Te Velthuis, and E. Fodor. 2017. Role of the PB2 627 Domain in Influenza A Virus Polymerase Function. *J Virol*. 91:e00523-00517.

Nobusawa, E., T. Aoyama, H. Kato, Y. Suzuki, Y. Tateno, and K. Nakajima. 1991. Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. *Virology*. 182:475-485.

Noton, S.L., E. Medcalf, D. Fisher, A.E. Mullin, D. Elton, and P. Digard. 2007. Identification of the domains of the influenza A virus M1 matrix protein required for NP binding, oligomerization and incorporation into virions. *J Gen Virol*. 88:2280-2290.

Ostrowsky, B., A. Huang, W. Terry, D. Anton, B. Brunagel, L. Traynor, S. Abid, G. Johnson, M. Kacica, J. Katz, L. Edwards, S. Lindstrom, A. Klimov, and T.M. Uyeki. 2012. Low pathogenic avian influenza A (H7N2) virus infection in immunocompromised adult, New York, USA, 2003. *Emerg Infect Dis*. 18:1128-1131.

Pantin-Jackwood, M., D.E. Swayne, D. Smith, and E. Shepherd. 2013. Effect of species, breed and route of virus inoculation on the pathogenicity of H5N1 highly pathogenic influenza (HPAI) viruses in domestic ducks. *Vet Res*. 44:62.

Pantin-Jackwood, M.J., M. Costa-Hurtado, K. Bertran, E. DeJesus, D. Smith, and D.E. Swayne. 2017a. Infectivity, transmission and pathogenicity of H5 highly pathogenic avian influenza clade 2.3.4.4 (H5N8 and H5N2) United States index viruses in Pekin ducks and Chinese geese. *Vet Res*. 48:33.

Pantin-Jackwood, M.J., M. Costa-Hurtado, E. Shepherd, E. DeJesus, D. Smith, E. Spackman, D.R. Kapczynski, D.L. Suarez, D.E. Stallknecht, and D.E. Swayne. 2016. Pathogenicity and Transmission of H5 and H7 Highly Pathogenic Avian Influenza Viruses in Mallards. *J Virol*. 90:9967-9982.

Pantin-Jackwood, M.J., D.M. Smith, J.L. Wasilenko, C. Cagle, E. Shepherd, L. Sarmento, D.R. Kapczynski, and C.L. Afonso. 2012. Effect of age on the pathogenesis and innate immune responses in Pekin ducks infected with different H5N1 highly pathogenic avian influenza viruses. *Virus Res*. 167:196-206.

Pantin-Jackwood, M.J., C.B. Stephens, K. Bertran, D.E. Swayne, and E. Spackman. 2017b. The pathogenesis of H7N8 low and highly pathogenic avian influenza viruses from the United States 2016 outbreak in chickens, turkeys and mallards. *PLoS One*. 12:e0177265.

Pantin-Jackwood, M.J., and D.E. Swayne. 2007. Pathobiology of Asian highly pathogenic avian influenza H5N1 virus infections in ducks. *Avian Dis*. 51:250-259.

Pantin-Jackwood, M.J., and D.E. Swayne. 2009. Pathogenesis and pathobiology of avian influenza virus infection in birds. *Revue scientifique et technique*. 28:113-136.

Park, S., J. Il Kim, I. Lee, J.Y. Bae, K. Yoo, M. Nam, J. Kim, M. Sook Park, K.J. Song, J.W. Song, S.H. Kee, and M.S. Park. 2017. Adaptive mutations of neuraminidase stalk truncation and deglycosylation confer enhanced pathogenicity of influenza A viruses. *Sci Rep*. 7:10928.

Parrish, C.R., and I.E.H. Voorhees. 2019. H3N8 and H3N2 Canine Influenza Viruses: Understanding These New Viruses in Dogs. *Vet Clin North Am Small Anim Pract*. 49:643-649.

Pasick, J., Y. Berhane, T. Hisanaga, H. Kehler, K. Hooper-McGrevy, K. Handel, J. Neufeld, C. Argue, and F. Leighton. 2010. Diagnostic test results and pathology associated with the 2007 Canadian H7N3 highly pathogenic avian influenza outbreak. *Avian Dis*. 54:213-219.

Pasick, J., K. Handel, J. Robinson, J. Copps, D. Ridd, K. Hills, H. Kehler, C. Cottam-Birt, J. Neufeld, Y. Berhane, and S. Czub. 2005. Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. *J Gen Virol*. 86:727-731.

Perdue, M., J. Crawford, M. Garcia, J. Latimer, and D.E. Swayne. 2003. Occurrence and Possible Mechanisms of Cleavage-Site Insertions in the Avian Influenza Hemagglutinin Gene. *Avian Diseases*. 47 Special Issue, Fourth International Symposium on Avian Influenza, 1997 Proceedings (2003):182-193.

Petersen, H., Z. Wang, E. Lenz, S. Pleschka, and S. Rautenschlein. 2013. Reassortment of NS segments modifies highly pathogenic avian influenza virus interaction with avian hosts and host cells. *J Virol*. 87:5362-5371.

Pflug, A., D. Guilligay, S. Reich, and S. Cusack. 2014. Structure of influenza A polymerase bound to the viral RNA promoter. *Nature*. 516:355-360.

Pflug, A., M. Lukarska, P. Resa-Infante, S. Reich, and S. Cusack. 2017. Structural insights into RNA synthesis by the influenza virus transcription-replication machine. *Virus Res*. 234:103-117.

Pichlmair, A., O. Schulz, C.P. Tan, T.I. Naslund, P. Liljestrom, F. Weber, and C. Reis e Sousa. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science*. 314:997-1001.

Pillai, S.P., M. Pantin-Jackwood, H.M. Yassine, Y.M. Saif, and C.W. Lee. 2010. The high susceptibility of turkeys to influenza viruses of different origins implies their importance as potential intermediate hosts. *Avian Dis*. 54:522-526.

Probst, C., J.M. Gethmann, H.J. Petermann, J. Neudecker, K. Jacobsen, and F.J. Conraths. 2012. Low pathogenic avian influenza H7N7 in domestic poultry in Germany in 2011. *Vet Rec*. 171:624.

Puzelli, S., L. Di Trani, C. Fabiani, L. Campitelli, M.A. De Marco, I. Capua, J.F. Aguilera, M. Zambon, and I. Donatelli. 2005. Serological analysis of serum samples from humans exposed to avian H7 influenza viruses in Italy between 1999 and 2003. *The Journal of infectious diseases*. 192:1318-1322.

Puzelli, S., G. Rossini, M. Facchini, G. Vaccari, L. Di Trani, A. Di Martino, P. Gaibani, C. Vocale, G. Cattoli, M. Bennett, J.W. McCauley, G. Rezza, M.L. Moro, R. Rangoni, A.C. Finarelli, M.P. Landini, M.R. Castrucci, I. Donatelli, and F. Influenza Task. 2014. Human infection with highly pathogenic A(H7N7) avian influenza virus, Italy, 2013. *Emerg Infect Dis*. 20:1745-1749.

Racaniello, V. 2009. Structure of influenza virus http://www.virology.ws/2009/04/30/structure-of-influenza-virus/ last accessed on 15.06.19.

Reid, A.H., and J.K. Taubenberger. 2003. The origin of the 1918 pandemic influenza virus: a continuing enigma. *J Gen Virol*. 84:2285-2292.

Rojas, H., R. Moreira, P. Avalos, I. Capua, and S. Marangon. 2002. Avian influenza in poultry in Chile. *Vet Rec*. 151:188.

Rust, M.J., M. Lakadamyali, F. Zhang, and X. Zhuang. 2004. Assembly of endocytic machinery around individual influenza viruses during viral entry. *Nat Struct Mol Biol*. 11:567-573.

Samji, T. 2009. Influenza A: understanding the viral life cycle. *Yale J Biol Med*. 82:153-159.

Sarmento, L., J. Wasilenko, and M. Pantin-Jackwood. 2010. The effects of NS gene exchange on the pathogenicity of H5N1 HPAI viruses in ducks. *Avian Dis*. 54:532-537.

Schafer, W., A. Stroh, S. Berghofer, J. Seiler, M. Vey, M.L. Kruse, H.F. Kern, H.D. Klenk, and W. Garten. 1995. Two independent targeting signals in the cytoplasmic domain determine trans-Golgi network localization and endosomal trafficking of the proprotein convertase furin. *EMBO J*. 14:2424-2435.

Scheibner, D., C. Blaurock, T.C. Mettenleiter, and E.M. Abdelwhab. 2019a. Virulence of three European highly pathogenic H7N1 and H7N7 avian influenza viruses in Pekin and Muscovy ducks. *BMC Vet Res*. 15:142.

Scheibner, D., R. Ulrich, O.I. Fatola, A. Graaf, M. Gischke, A.H. Salaheldin, T.C. Harder, J. Veits, T.C. Mettenleiter, and E.M. Abdelwhab. 2019b. Variable impact of the hemagglutinin polybasic cleavage site on virulence and pathogenesis of avian influenza H7N7 virus in chickens, turkeys and ducks. *Sci Rep*. 9:11556.

Schlee, M., A. Roth, V. Hornung, C.A. Hagmann, V. Wimmenauer, W. Barchet, C. Coch, M. Janke, A. Mihailovic, G. Wardle, S. Juranek, H. Kato, T. Kawai, H. Poeck, K.A. Fitzgerald, O. Takeuchi, S. Akira, T. Tuschl, E. Latz, J. Ludwig, and G. Hartmann. 2009.

Recognition of 5' triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity*. 31:25-34.

Schmitt, A.P., and R.A. Lamb. 2005. Influenza virus assembly and budding at the viral budozone. *Adv Virus Res*. 64:383-416.

Schmolke, M., B. Manicassamy, L. Pena, T. Sutton, R. Hai, Z.T. Varga, B.G. Hale, J. Steel, D.R. Perez, and A. Garcia-Sastre. 2011. Differential contribution of PB1-F2 to the virulence of highly pathogenic H5N1 influenza A virus in mammalian and avian species. *PLoS Pathog*. 7:e1002186.

Schotsaert, M., and A. Garcia-Sastre. 2014. Influenza vaccines: a moving interdisciplinary field. *Viruses*. 6:3809-3826.

Seekings, A.H., M.J. Slomka, C. Russell, W.A. Howard, B. Choudhury, A. Nunez, B.Z. Londt, W. Cox, V. Ceeraz, P. Thoren, R.M. Irvine, R.J. Manvell, J. Banks, and I.H. Brown. 2018. Direct evidence of H7N7 avian influenza virus mutation from low to high virulence on a single poultry premises during an outbreak in free range chickens in the UK, 2008. *Infect Genet Evol*. 64:13-31.

Selman, M., S.K. Dankar, N.E. Forbes, J.J. Jia, and E.G. Brown. 2012. Adaptive mutation in influenza A virus non-structural gene is linked to host switching and induces a novel protein by alternative splicing. *Emerg Microbes Infect*. 1:e42.

Shao, W., X. Li, M.U. Goraya, S. Wang, and J.L. Chen. 2017. Evolution of Influenza A Virus by Mutation and Re-Assortment. *Int J Mol Sci*. 18.

Sims, L.D., J. Weaver, and D.E. Swayne. 2016. Epidemiology of avian influenza in agricultural and other man-made systems. *In* Animal influenza 2nd edition.

Skowronski, D.M., S.A. Tweed, M. Petric, T. Booth, Y. Li, and T. Tam. 2006. Human illness and isolation of low-pathogenicity avian influenza virus of the H7N3 subtype in British Columbia, Canada. *The Journal of infectious diseases*. 193:899-900; author reply 900-891.

Slemons, R.D., and B.C. Easterday. 1978. Virus replication in the digestive tract of ducks exposed by aerosol to type-A influenza. *Avian Dis*. 22:367-377.

Slemons, R.D., D.C. Johnson, J.S. Osborn, and F. Hayes. 1974. Type-A influenza viruses isolated from wild free-flying ducks in California. *Avian Dis*. 18:119-124.

Slomka, M.J., A. Puranik, S. Mahmood, S.S. Thomas, A.H. Seekings, A.M.P. Byrne, A. Nunez, C. Bianco, B.C. Mollett, S. Watson, I.H. Brown, and S.M. Brookes. 2018. Ducks Are Susceptible to Infection with a Range of Doses of H5N8 Highly Pathogenic Avian Influenza Virus (2016, Clade 2.3.4.4b) and Are Largely Resistant to Virus-Specific Mortality, but Efficiently Transmit Infection to Contact Turkeys. *Avian Dis*. 63:172-180.

Soliman, A., M. Saad, E. Elassal, E. Amir, C. Plathonoff, V. Bahgat, M. El-Badry, L.S. Ahmed, M. Fouda, M. Gamaleldin, N.A. Mohamed, S. Salyer, C. Cornelius, and R. Barthel.

2012. Surveillance of avian influenza viruses in migratory birds in Egypt, 2003-09. *J Wildl Dis*. 48:669-675.

Song, J., H. Feng, J. Xu, D. Zhao, J. Shi, Y. Li, G. Deng, Y. Jiang, X. Li, P. Zhu, Y. Guan, Z. Bu, Y. Kawaoka, and H. Chen. 2011. The PA protein directly contributes to the virulence of H5N1 avian influenza viruses in domestic ducks. *J Virol*. 85:2180-2188.

Soubies, S.M., T.W. Hoffmann, G. Croville, T. Larcher, M. Ledevin, D. Soubieux, P. Quere, J.L. Guerin, D. Marc, and R. Volmer. 2013. Deletion of the C-terminal ESEV domain of NS1 does not affect the replication of a low-pathogenic avian influenza virus H7N1 in ducks and chickens. *J Gen Virol*. 94:50-58.

Stech, O., J. Veits, S.M. Abdelwhab el, U. Wessels, T.C. Mettenleiter, and J. Stech. 2015. The Neuraminidase Stalk Deletion Serves as Major Virulence Determinant of H5N1 Highly Pathogenic Avian Influenza Viruses in Chicken. *Sci Rep*. 5:13493.

Stech, O., J. Veits, S. Weber, D. Deckers, D. Schroer, T.W. Vahlenkamp, A. Breithaupt, J. Teifke, T.C. Mettenleiter, and J. Stech. 2009. Acquisition of a polybasic hemagglutinin cleavage site by a low-pathogenic avian influenza virus is not sufficient for immediate transformation into a highly pathogenic strain. *J Virol*. 83:5864-5868.

Steinhauer, D.A. 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. *Virology*. 258:1-20.

Stevaert, A., and L. Naesens. 2016. The Influenza Virus Polymerase Complex: An Update on Its Structure, Functions, and Significance for Antiviral Drug Design. *Med Res Rev*. 36:1127-1173.

Stieneke-Grober, A., M. Vey, H. Angliker, E. Shaw, G. Thomas, C. Roberts, H.D. Klenk, and W. Garten. 1992. Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. *EMBO J*. 11:2407-2414.

Stucker, K.M., S.A. Schobel, R.J. Olsen, H.L. Hodges, X. Lin, R.A. Halpin, N. Fedorova, T.B. Stockwell, A. Tovchigrechko, S.R. Das, D.E. Wentworth, and J.M. Musser. 2015. Haemagglutinin mutations and glycosylation changes shaped the 2012/13 influenza A(H3N2) epidemic, Houston, Texas. *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin*. 20.

Suarez, D.L., M. Garcia, J. Latimer, D. Senne, and M. Perdue. 1999. Phylogenetic analysis of H7 avian influenza viruses isolated from the live bird markets of the Northeast United States. *J Virol*. 73:3567-3573.

Suarez, D.L., D.A. Senne, J. Banks, I.H. Brown, S.C. Essen, C.W. Lee, R.J. Manvell, C. Mathieu-Benson, V. Moreno, J.C. Pedersen, B. Panigrahy, H. Rojas, E. Spackman, and D.J. Alexander. 2004. Recombination resulting in virulence shift in avian influenza outbreak, Chile. *Emerg Infect Dis*. 10:693-699.

Sutton, T.C. 2018. The Pandemic Threat of Emerging H5 and H7 Avian Influenza Viruses. *Viruses*. 10:461.

Sutton, T.C., C. Finch, H. Shao, M. Angel, H. Chen, I. Capua, G. Cattoli, I. Monne, and D.R. Perez. 2014. Airborne transmission of highly pathogenic H7N1 influenza virus in ferrets. *J Virol*. 88:6623-6635.

Tatulian, S.A., P. Hinterdorfer, G. Baber, and L.K. Tamm. 1995. Influenza hemagglutinin assumes a tilted conformation during membrane fusion as determined by attenuated total reflection FTIR spectroscopy. *EMBO J*. 14:5514-5523.

Thanawongnuwech, R., A. Amonsin, R. Tantilertcharoen, S. Damrongwatanapokin, A. Theamboonlers, S. Payungporn, K. Nanthapornphiphat, S. Ratanamungklanon, E. Tunak, T. Songserm, V. Vivatthanavanich, T. Lekdumrongsak, S. Kesdangsakonwut, S. Tunhikorn, and Y. Poovorawan. 2005. Probable tiger-to-tiger transmission of avian influenza H5N1. *Emerg Infect Dis*. 11:699-701.

Tong, X.C., S.S. Weng, F. Xue, X. Wu, T.M. Xu, and W.H. Zhang. 2018. First human infection by a novel avian influenza A(H7N4) virus. *J Infect*. 77:249-257.

Tweed, S.A., D.M. Skowronski, S.T. David, A. Larder, M. Petric, W. Lees, Y. Li, J. Katz, M. Krajden, R. Tellier, C. Halpert, M. Hirst, C. Astell, D. Lawrence, and A. Mak. 2004. Human illness from avian influenza H7N3, British Columbia. *Emerg Infect Dis*. 10:2196-2199.

van Kolfschooten, F. 2003. Dutch veterinarian becomes first victim of avian influenza. *Lancet*. 361:1444.

Varghese, J.N., W.G. Laver, and P.M. Colman. 1983. Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 A resolution. *Nature*. 303:35-40.

Varki, A. 2008. Sialic acids in human health and disease. *Trends Mol Med*. 14:351-360.

Veits, J., S. Weber, O. Stech, A. Breithaupt, M. Graber, S. Gohrbandt, J. Bogs, J. Hundt, J.P. Teifke, T.C. Mettenleiter, and J. Stech. 2012. Avian influenza virus hemagglutinins H2, H4, H8, and H14 support a highly pathogenic phenotype. *Proc Natl Acad Sci U S A*. 109:2579-2584.

Verbraucherschutz, B.f.J.u. 2019. Geflügelpestschutzverordnung https://www.gesetze-im-internet.de/geflpestschv/BJNR234800007.html last accessed on 20.06.19.

Viruses, I.C.o.T.o., and ICTV. 2018. https://talk.ictvonline.org/ictv-reports/ictv_9th_report/negative-sense-rna-viruses-2011/w/negrna_viruses/209/orthomyxoviridae last accessed on 28.07.19.

Wagner, R., M. Matrosovich, and H.D. Klenk. 2002. Functional balance between haemagglutinin and neuraminidase in influenza virus infections. *Rev Med Virol*. 12:159-166.

Wagner, R., T. Wolff, A. Herwig, S. Pleschka, and H.D. Klenk. 2000. Interdependence of hemagglutinin glycosylation and neuraminidase as regulators of influenza virus growth: a study by reverse genetics. *J Virol*. 74:6316-6323.

Wang, C.C., J.R. Chen, Y.C. Tseng, C.H. Hsu, Y.F. Hung, S.W. Chen, C.M. Chen, K.H. Khoo, T.J. Cheng, Y.S. Cheng, J.T. Jan, C.Y. Wu, C. Ma, and C.H. Wong. 2009. Glycans on influenza hemagglutinin affect receptor binding and immune response. *Proc Natl Acad Sci U S A*. 106:18137-18142.

Wang, N., M. Sun, W. Wang, G. Ouyang, Z. Chen, Y. Zhang, B. Zhao, S. Wu, J. Huang, H. Sun, M. Liao, and P. Jiao. 2017. Avian Influenza (H7N9) Viruses Co-circulating among Chickens, Southern China. *Emerg Infect Dis*. 23:2100-2102.

Webster, R.G., W.J. Bean, O.T. Gorman, T.M. Chambers, and Y. Kawaoka. 1992. Evolution and ecology of influenza A viruses. *Microbiol Rev*. 56:152-179.

Webster, R.G., J. Geraci, G. Petursson, and K. Skirnisson. 1981. Conjunctivitis in human beings caused by influenza A virus of seals. *N Engl J Med*. 304:911.

Webster, R.G., W.G. Laver, and E.D. Kilbourne. 1968. Reactions of antibodies with surface antigens of influenza virus. *J Gen Virol*. 3:315-326.

Webster, R.G., M. Yakhno, V.S. Hinshaw, W.J. Bean, and K.G. Murti. 1978. Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology*. 84:268-278.

Wei, L., P. Jiao, Y. Song, L. Cao, R. Yuan, L. Gong, J. Cui, S. Zhang, W. Qi, S. Yang, and M. Liao. 2013. Host immune responses of ducks infected with H5N1 highly pathogenic avian influenza viruses of different pathogenicities. *Vet Microbiol*. 166:386-393.

Werner, O., E. Starick, and C.H. Grund. 2003. Isolation and characterization of a low-pathogenicity H7N7 influenza virus from a turkey in a small mixed free-range poultry flock in Germany. *Avian Dis*. 47:1104-1106.

Wessels, U., E.M. Abdelwhab, J. Veits, D. Hoffmann, S. Mamerow, O. Stech, J. Hellert, M. Beer, T.C. Mettenleiter, and J. Stech. 2018. A Dual Motif in the Hemagglutinin of H5N1 Goose/Guangdong-Like Highly Pathogenic Avian Influenza Virus Strains Is Conserved from Their Early Evolution and Increases both Membrane Fusion pH and Virulence. *J Virol*. 92:00778-00718.

Wharton, S.A., R.B. Belshe, J.J. Skehel, and A.J. Hay. 1994. Role of virion M2 protein in influenza virus uncoating: specific reduction in the rate of membrane fusion between virus and liposomes by amantadine. *J Gen Virol*. 75:945-948.

WHO. 1980. A revision of the system of nomenclature for influenza viruses: a WHO memorandum. *Bulletin of the World Health Organization*. 58:585-591.

Wiley, D.C., and J.J. Skehel. 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu Rev Biochem*. 56:365-394.

Wise, H.M., A. Foeglein, J. Sun, R.M. Dalton, S. Patel, W. Howard, E.C. Anderson, W.S. Barclay, and P. Digard. 2009. A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. *J Virol*. 83:8021-8031.

Wise, H.M., E.C. Hutchinson, B.W. Jagger, A.D. Stuart, Z.H. Kang, N. Robb, L.M. Schwartzman, J.C. Kash, E. Fodor, A.E. Firth, J.R. Gog, J.K. Taubenberger, and P. Digard. 2012. Identification of a novel splice variant form of the influenza A virus M2 ion channel with an antigenically distinct ectodomain. *PLoS Pathog*. 8:e1002998.

Xue, R., Y. Tian, T. Hou, D. Bao, H. Chen, Q. Teng, J. Yang, X. Li, G. Wang, Z. Li, and Q. Liu. 2018. H9N2 influenza virus isolated from minks has enhanced virulence in mice. *Transboundary and emerging diseases*. 65:904-910.

Yamayoshi, S., M. Watanabe, H. Goto, and Y. Kawaoka. 2016. Identification of a Novel Viral Protein Expressed from the PB2 Segment of Influenza A Virus. *J Virol*. 90:444-456.

Yang, L., W. Zhu, X. Li, M. Chen, J. Wu, P. Yu, S. Qi, Y. Huang, W. Shi, J. Dong, X. Zhao, W. Huang, Z. Li, X. Zeng, H. Bo, T. Chen, W. Chen, J. Liu, Y. Zhang, Z. Liang, W. Shi, Y. Shu, and D. Wang. 2017. Genesis and Spread of Newly Emerged Highly Pathogenic H7N9 Avian Viruses in Mainland China. *J Virol*. 91:01277-01217.

Yasuhara, A., S. Yamayoshi, M. Kiso, Y. Sakai-Tagawa, M. Koga, E. Adachi, T. Kikuchi, I.H. Wang, S. Yamada, and Y. Kawaoka. 2019. Antigenic drift originating from changes to the lateral surface of the neuraminidase head of influenza A virus. *Nat microbiol*. 4:1024–1034.

Ye, Q., R.M. Krug, and Y.J. Tao. 2006. The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA. *Nature*. 444:1078-1082.

Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, and T. Fujita. 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol*. 5:730-737.

Zanella, A., P. Dall'Ara, and P.A. Martino. 2001. Avian influenza epidemic in Italy due to serovar H7N1. *Avian Dis*. 45:257-261.

Zhong, G., M.Q. Le, T.J.S. Lopes, P. Halfmann, M. Hatta, S. Fan, G. Neumann, and Y. Kawaoka. 2018. Mutations in the PA Protein of Avian H5N1 Influenza Viruses Affect Polymerase Activity and Mouse Virulence. *J Virol*. 92.

Zhou, H., J. Zhu, J. Tu, W. Zou, Y. Hu, Z. Yu, W. Yin, Y. Li, A. Zhang, Y. Wu, Z. Yu, H. Chen, and M. Jin. 2010. Effect on virulence and pathogenicity of H5N1 influenza A virus through truncations of NS1 eIF4GI binding domain. *The Journal of infectious diseases*. 202:1338-1346.

9 List of Publications

9.1 Publications of the presented data

9.1.1 Articles in scientific journals

David Scheibner, Reiner Ulrich, Olanrewaju I. Fatola, Annika Graaf, Marcel Gischke, Ahmed H. Salaheldin, Timm C. Harder, Jutta Veits, Thomas C. Mettenleiter and Elsayed M. Abdelwhab.

The impact of the hemagglutinin polybasic cleavage site on the virulence of avian influenza H7N7 virus in chickens, turkeys and ducks.

Scientific Reports, published on 09.08.19, 9:11556.

David Scheibner, Claudia Blaurock, Thomas C. Mettenleiter, Elsayed M. Abdelwhab.

Virulence of three European highly pathogenic H7N1 and H7N7 avian influenza viruses in Pekin and Muscovy ducks.

BMC Veterinary Research, published on 10.05.19, 15:142

9.1.2 Abstracts

David Scheibner, Jutta Veits, El-Sayed M. Abdelwhab and Thomas C. Mettenleiter (2016).

Virulence determinants of recent avian influenza viruses of subtype H7N7 in chickens.

Junior Scientist Symposium, 21.-23. September,Jena, Germany (Poster)

David Scheibner, Jutta Veits, Thomas C. Mettenleiter and El-Sayed M. Abdelwhab (2017).

Virulence determinants of a recent H7N7 avian influenza virus in chickens.

The 27th Annual Meeting of the German Society for Virology, 22 -25 March, Marburg, Germany (Poster).

David Scheibner, Jutta Veits, Thomas C. Mettenleiter and El-Sayed M. Abdelwhab. (2017)

Variable pathogenicity of H7N7 recombinant viruses in chickens, turkeys and ducks.

The 6th ESWI Influenza Conference, 10-13 September Riga, Latvia (Oral Presentation)

David Scheibner, Jutta Veits, Thomas C. Mettenleiter and El-Sayed M. Abdelwhab. (2017).

Virulence determinants of recent German avian influenza H7N7 viruses in different poultry species
Junior Scientist Symposium, 20-22 September, Braunschweig (Oral Presentation)

David Scheibner, Ahmed H. Salaheldin, Felix Winter, Marcel Gischke, Jutta Veits, Thomas C. Mettenleiter and Elsayed M. Abdelwhab (2017)
Virulence determinants of recent German avian influenza isolates subtype H7N7 in different host species
1st International conference on Respiratory Pathogens, 1-3 November Rostock, Germany (Oral Presentation)

David Scheibner, Ahmed H. Salaheldin, Marcel Gischke, Felix Winter, Jutta Veits, Thomas C. Mettenleiter and El-Sayed M. Abdelwhab (2018).
Virulence determinants of a recent H7N7 avian influenza virus in chickens.
The 28th Annual Meeting of the German Society for Virology, 14-17 March, Würzburg, Germany (Poster).

David Scheibner, Ahmed H. Slaheldin, Felix Winter, Marcel Gischke, Jutta Veits, Thomas C. Mettenleiter and Elsayed M. Abdelwhab (2018)
Pathogenicity and virulence determinants of recent German H7N7 viruses in different poultry species.
10th International Symposium on Avian Influenza, 15-18 April, Brighton, England (Oral Presentation)

David Scheibner, Jutta Veits, Thomas C. Mettenleiter and El-Sayed M. Abdelwhab. (2018).
Virulence determinants of recent German avian influenza H7N7 viruses in different poultry species
Junior Scientist Symposium, 24-26 September, Greifswald-Riems, Germany (Poster)

David Scheibner, Jutta Veits, Thomas C. Mettenleiter and El-Sayed M. Abdelwhab (2019).
Impact of the polybasic cleavage site within the HA of a recent German H7N7 virus on its pathogenicity in different poultry species
The 29th Annual Meeting of the German Society for Virology, 20-23 March, Düsseldorf, Germany (Poster).

9.2 Other publications in scientific journals

Graaf A, Ulrich R, Maksimov P, **Scheibner D**, Koethe S, Abdelwhab EM, Mettenleiter TC, Beer M, Harder T.
A viral race for primacy: co-infection of a natural pair of low and highly pathogenic H7N7 avian influenza viruses in chickens and embryonated chicken eggs. Emerging Microbes and Infections, 2018, 7:204

Salaheldin AH, Kasbohm E, El-Naggar H, Ulrich R, **Scheibner D**, Gischke M, Hassan MK, Arafa AA, Hassan WM, Abd El-Hamid HS, Hafez HM, Veits J, Mettenleiter TC, Abdelwhab EM. *Potential Biological and Climatic Factors That Influence the Incidence and Persistence of Highly Pathogenic H5N1 Avian Influenza Virus in Egypt.*
Frontiers in Microbiology, 2018, 9:528

Dittrich A, **Scheibner D**, Salaheldin AH, Veits J, Gischke M, Mettenleiter TC, Abdelwhab EM**.** *Impact of Mutations in the Hemagglutinin of H10N7 Viruses Isolated from Seals on Virus Replication in Avian and Human Cells.*
Viruses, 2018, 10(2):83

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12 Declaration Freie Universität Berlin

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Detaillierte Einschätzung der Beteiligung von David Scheibner an den Publikationen, die im Rahmen dieser kumulativen Dissertation präsentiert wurden:

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