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From low to high pathogenicity –
Characterization of avian influenza viruses of subtypes H7 and H5

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CHAPTER I: INTRODUCTION

I. INTRODUCTION

During the last decade, the emergence and spread of new avian influenza viruses (AIV) have caused huge economic losses in poultry production worldwide and have adversely affected wild bird populations (OIE, 2018a). While the infection with low pathogenic (LP) AIV is widespread in wild birds and leads, when transmitted to poultry, to sporadic outbreaks with no serious concern for animal health, the potential emergence and spread of highly pathogenic (HP) AIV from LPAIV precursor viruses has a high negative impact on poultry production (Dhingra et al., 2018; Spackman, 2008). Among all AIV, only viruses of the subtypes H5 and H7 have the natural potential to mutate spontaneously from LP into HP phenotypes (Capua et al., 2013b; Deshpande et al., 1987). Apart from causing devastating losses in poultry, due to the zoonotic propensity of some of these viruses, they also pose a threat to public health (Kalthoff et al., 2010).

The described natural history of AIV dates back to 1878 in Northern Italy (Perroncito, 1878). Although the first documented isolation of influenza virus of subtype H7 (A/Chicken/Brescia/1902 (H7N7) was made in 1902 in Italy (Horimoto et al., 2001), it was not before 1955 that Schäfer characterized these agents as influenza A viruses (Schäfer, 1955). AIV of subtype H7N7 is still endemic in wild birds in Europe and is rarely though regularly isolated also from domestic poultry (Abdelwhab et al., 2014b). Moreover, several strains of AIV H7 also pose a threat to human health and have led to several sporadic mild to fatal infections in humans (Wong et al., 2006). Highly zoonotic LPAIV H7N9 surfaced in China in 2013 and since then spread in at least five waves among poultry populations across the country with spill-over transmissions to humans which resulted in 1625 confirmed human infections and 623 deaths (FAO, 2018; Li et al., 2014; Shi et al., 2017).

Further, subtype H5 viruses of low pathogenicity are endemic in migratory wild bird populations and potentially mutate into HPAIV strains following transmission into poultry (Swayne et al., 2000). The currently most widespread HPAIV lineage of subtype H5 emerged from geese in the Chinese Guangdong province in 1996 and led since then to unprecedented global spread and mortality in wild bird species and poultry as well as to 860 human cases and 454 deaths (Peiris et al., 2007; WHO, 2018b).

Determining the drivers of emergence and spread of HPAIV is crucial for a better understanding why and when certain LP strains pose a risk of evolving to HP. Although there are various hypotheses describing the emergence of HPAIV from LPAIV precursors (Richard et al., 2017), there is insufficient knowledge why the HP phenotype emerges (naturally) only in H5 and H7 subtypes. It is also unclear how the HP variant escapes from the index bird which necessarily is infected by the LP precursor. So far, only a few cases worldwide have been reported in which both the LPAI precursor virus and the descant HPAIV strain (i.e. a natural “matching LP/HPAI virus pair”) were detected and epidemiologically linked (Dhingra et al., 2018; Wood JM, 1985).

Therefore, the present study is aiming at casting some light on the mechanisms by which HPAIV (i) emerge, (ii) initially spread in the index bird and (iii) are transmitted to in-contact birds.

CHAPTER II: REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

1. AVIAN INFLUENZA VIRUS

Avian influenza (AI) is an infectious viral disease of birds caused by influenza A viruses (Lupiani et al., 2009; Swayne et al., 2000; Thomas et al., 2007). Metapopulations of wild waterfowl represent the primary reservoir of AIV from where the virus can be transmitted to domestic poultry (Alexander et al., 2009; Webster et al., 1992a). Some virus strains may also be of concern for mammalians including humans (Freidl et al., 2014; Herfst et al., 2014; Webster et al., 1992a).

1.1 Virus taxonomy

AIV belong to the family *Orthomyxoviridae* that contains *s*even different genera of influenza viruses: Influenza A, B, C and D viruses, Thogoto virus, aquatic infectious salmon anemia virus and Quaranjavirus (Hause et al., 2014b; ICTV, 2017; Presti et al., 2009). AIV themselves are members of the influenza A virus (IAV) genus and, based on the antigenic variation of the two integral major surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), can theoretically be classified into 154 different subtypes (Bouvier et al., 2008). Eighteen subtypes of HA (H1-H18) and eleven subtypes of NA (N1-N11) have been identified so far (Hause et al., 2014a; ICTV, 2017; Schrauwen et al., 2014). While H1-H16 have been described in waterfowl (Spackman et al., 2003), H17N10 and H18N11 have been found in bats only (Mehle, 2014; Tong et al., 2013b). IAV can further be distinguished into several lineages according to their main host reservoirs including human, swine, equine, and avian influenza viruses (Gorman et al., 1990) and bat influenza viruses (Tong et al., 2013a). According to their pathogenicity in chickens, AIV can be categorized into two phenotypes: Low pathogenic (LP) and highly pathogenic (HP) AIV. While LPAIV cause no or only mild clinical signs in birds, HPAIV can cause up to 100% morbidity and mortality within a few days (Alexander, 2000). A standard nomenclature for influenza viruses was established in February 1980 by the World Health Organization (WHO): This supplies information on the influenza type A, B or C, the host of origin (for non-human viruses), geographical origin, strain number (laboratory number/code), year of isolation and the HA/NA subtype (e.g.

A/chicken/Germany/AR915/2015 (H7N7)) (Assaad et al., 1980). In case of human-derived viruses, the host origin is omitted (e.g. A/Germany/767/95 (H3N2)) (IRD, 2018).

1.2. Virion structure

IAV, including those of avian origin, are polymorphic in shape and appear as either spherical (about 100-120 nm in diameter) or filamentous (with a length of up to 1000 nm) particles (Samji, 2009). The virus particle contains a lipid bilayer envelope obtained from the host cell membrane after egress by budding. IAV harbor a negative-sensed, single-stranded (ss), encapsidated ribonucleic acid (RNA) eight-fold segmented genome of about 13.5 kilo base pairs (kbp) in total. Two major surface glycoproteins, the HA and NA, are anchored within the virus membrane. Another surface protein, the matrix-2 (M2) protein, forms a transmembrane ion channel. In addition to this, six internal proteins comprising of polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), nucleoprotein (NP), matrix protein (M) and non-structural protein 2 (NS2) are present in the virion.

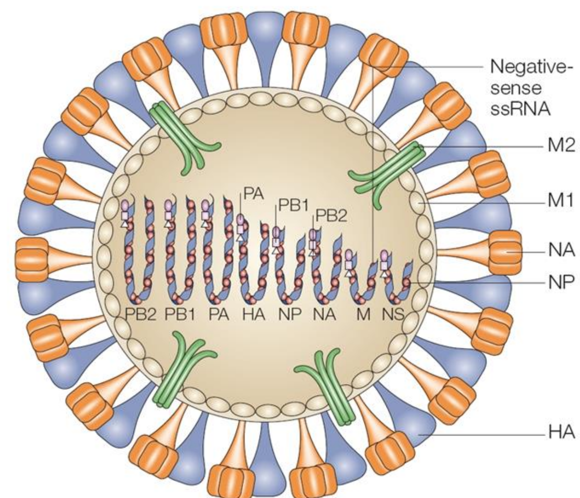

Figure 1. Structure of influenza A virus (Horimoto et al., 2005)*.

* For permission rights see chapter IX. Supplement, page 188.

1.3. Genome organization

The genome of AIV contains all genetic information necessary for replication and assembly of progeny virions (Brown, 2000) and is composed of eight gene segments (designated PB2, PB1, PA, HA, NP, NA, M, NS) that are numbered in order of decreasing length. These segments hold coding capacities for nine structural proteins (surface and/or internal proteins PB2, PB1, PA, HA, NP, NA, M1, M2, NS2) and up to five non-structural proteins which have been identified in infected cells but not in virions (PB2-S1, PB1-F2, PB1-N40, PA-X and NS1; (Alexander et al., 2009; Nayak, 1969; Nayak et al., 2009; Webster et al., 1992a). The encoded polypeptides and their respective functions are summarized in Table 1.

Critical for the formation of the ribonucleoprotein complex (RNP) and for the binding of the polymerase complex (composed of each one copy of the PB2, PB1 and PA proteins), all gene segments harbor 12 highly conserved nucleotides in the 3´ terminus and 13 nucleotides in the 5´ terminus which are partially complementary and form a short double-stranded (ds) RNA structure known as the "panhandle" (Crescenzo-Chaigne et al., 2013; Fodor et al., 1994). Further, each gene segment has a segment-specific packaging signal that is located in the noncoding and terminal coding regions of both 3´ and 5´ ends of each viral RNA (vRNA) to ensure packaging of one copy of each segment into mature virions (Brown, 2000; Rossman et al., 2011). However, this concept has recently been challenged as more than 90 % of the virions seem to portray a wrong number or wrong combination of genome segments (Suarez, 2016).

PB1, PB2 and PA are the three proteins forming the viral RNA-dependent RNA polymerase (RdRP) complex and are encoded on separate segments. While PB1 and PB2 are basic proteins (Bouvier et al., 2008; Eisfeld et al., 2015)), PA is an acidic protein and tightly associated with PB2 and PB1 to form a compact structure (Area et al., 2004).

Segment 1 encodes the PB2 protein which recognizes and binds the 5´cap structures of host cell viral messenger RNA (mRNA). That is used as a viral mRNA transcription primer via a cap-snatching mechanism which plays an important role during initiation of mRNA transcription (Dias et al., 2009). In addition to this, a newly discovered protein, PB2-S1, translated from a spliced PB2 mRNA, has been described to inhibit the RIG-I dependent signaling pathway *in vitro* (Yamayoshi et al., 2015).

The PB1 RNA polymerase subunit is encoded by segment 2. PB1 is responsible for binding to the terminal 5´ and 3´ ends of both vRNA and complementary RNA (cRNA) and thus initiates the process of genome transcription and replication. PB1 also encodes the PB1-F2 protein (Chen et al., 2001) known to act as a virulence factor affecting pathogenesis, viral dissemination and transmission (McAuley et al., 2007).

The PA protein is encoded by segment 3. Although no specific function has been detailed so far, mutations in the PA may affect both transcription and replication procedures (Fodor et al., 2002). PA associates with PB2 and PB1 in the cap snatching process, aids in the initiation of mRNA synthesis and also has proteolytic activity. PA-X is a protein encoded by an alternate open reading frame of the PA gene and affects host protein expression and pathogenicity by controlling host inflammation, cell differentiation, apoptosis and tissue remodeling (Hayashi et al., 2015; Jagger et al., 2012).

The HA, as one of the two major glycoproteins, is encoded by segment 4 and embedded in the lipid bilayer of the viral envelope as a type I transmembrane protein (Veit et al., 2011). This protein is synthesized as a precursor protein (HA0) and must undergo posttranslational endoproteolytic cleavage into HA1 and HA2 (which remain connected by disulphide linkage) by cellular proteases (Steinhauer, 1999) in order to be activated and to render progeny virions infectious. HA monomers form a homotrimer resulting in two structurally distinct domains, HA1 and HA2 (Figure 2). The HA1 domain forms a globular head comprised of anti-parallel ß-sheets and loops that contains the receptor binding domain (RBD) and mediates HA attachment to the host cell surface. The HA2 part, comprised mainly of α-helices, function as a stalk and also harbors the membrane spanning anchor. The HA2 amino-terminal region, also known as the fusion peptide, is responsible for membrane fusion of the viral envelope with the cell endosome (Mair et al., 2014).

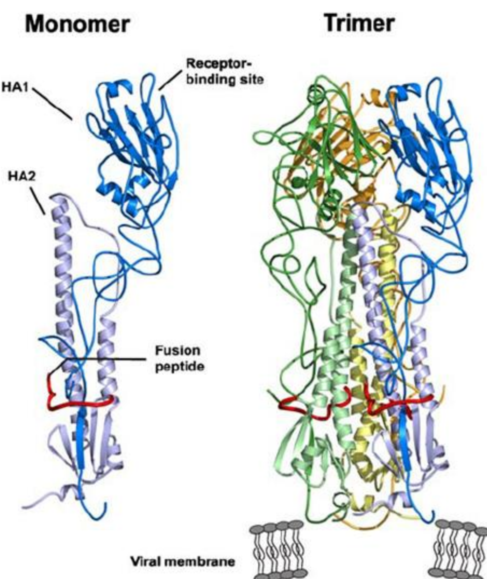

Figure 2. Structure of the hemagglutinin monomer (left) or trimer (right).
The endoproteolytic cleavage site (CS) is shown in dark red close to the fusion peptide (Amorij et al., 2008)*.

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The NP protein is encoded by segment 5 and is one of the essential components of the RNP complex (Cheung et al., 2007). As the amino terminus of the NP protein contains an RNA-binding domain, it has a structural role in binding and encapsidating vRNA through association with the polymerase proteins (Turrell et al., 2013).

The NA, also known as sialidase, is encoded by segment 6. NA is a type II transmembrane protein and encodes the most abundant surface glycoprotein, containing both antigenic and enzymatic properties (Gamblin et al., 2010). It is tetrameric, mushroom-shaped and bears cytoplasmic, transmembrane, stalk and head domains (Bouvier et al., 2008). NA, along with HA, plays an important role for the virulence of IAV. The NA head domain provides sialidase enzymatic activity. The main function of the NA is to cleave the sialic acid (SA) residue attached to the newly formed virions to release the viral particle from the plasma membrane during the budding process (Gamblin et al., 2010). This action also prevents self-aggregation and reattachment of the virus to the infected cell (Luo et al., 1993; Wagner et al., 2002). Epithelial cells in the respiratory tract secrete a mucus layer rich in sialylated glycoproteins, where IAV virions may easily get stuck by binding to such glycoproteins and never reach

sialylated receptors on the surface of the cell. In such cases it is believed that the NA protein cleaves those sialylated decoys and allows the virion to penetrate the mucus and gain entry into the host cell (Cohen et al., 2013).

The matrix (M) protein, encoded by segment 6, is the major structural component of the viral envelope, important for virus formation and induction of particle formation (Gomez-Puertas et al., 2000) and encodes two proteins: M1 and M2. Dimeric M1 forms a layer beneath the viral envelope stabilizing particle integrity. M1 interacts with the viral ribonucleoprotein (vRNP) complexes, forming a bridge between the inner core components and the membrane proteins (Boulo et al., 2007). The M2 protein is a homotetrameric integral membrane protein that functions as an ion channel and allows protons in the endosomal environment to flow into the virion during viral entry to the cell; M2 is encoded by a spliced M gene-derived mRNA (Manzoor et al., 2017; Moorthy et al., 2014). In addition to this, it has a role in genome packaging and in formation of virus particles (Chen et al., 2008).

Segment eight of the influenza genome codes for two major non-structural (NS) proteins. (Bouvier et al., 2008). The principle function of NS1 is suppressing the host antiviral response: NS1 has been shown to antagonize interferon (IFN) expression in infected cells by two pathways. The first one is binding to dsRNA (synthesized during viral replication), inhibiting the pre-transcription pathway for activation of IFN due to inactivation of cellular sensors such as protein kinase R (PKR), retinoic-acid inducible gene I (RIG-I) and 2´-5´ oligoadenylate synthetase-RNase (2´-5´OAS). In the second pathway, NS1 interacts with several IFN-induced cellular proteins/factors like cleavage and polyadenylation specificity factor 30 (CPSF30) and polyadenine binding protein II (PABPII) that are responsible for host mRNA maturation and export. Thus, post-transcriptional inhibition of IFN production occurs via binding of the NS1 protein to these proteins/factors that paralyses cellular gene expression and translation (Garcia-Sastre, 2001; Guo et al., 2007; Hatada et al., 1992; Krug, 2014; Min et al., 2006; Seo et al., 2012). The NS2 protein, renamed as nuclear export protein (NEP) after being demonstrated in the virion (Yasuda et al., 1993), is a structural protein which interacts with M1 protein (O'Neill et al., 1998) and is involved in the nuclear export of vRNPs and responsible for blocking the re-entry of vRNPs into nucleus (Eisfeld et al., 2015). More recently, a new protein product of the NS segment, NS3, was discovered and

tentatively described as a potential factor for adaptation of influenza A virus to mammalian (murine) hosts. (Selman et al., 2012).

Table 1. The gene segments of avian influenza virus and their encoded proteins (modified from (Bouvier et al., 2008; Naguib, 2017).

Segment	Name	Nucleotides (nt)	Encoded polypeptide	Protein length (aa)	Protein function
1	PB2	2341	PB2	759	Polymerase subunit; mRNA cap recognition; interacts with PA
			PB2-S1	510	inhibits the RIG-I dependent signalling pathway *in vitro*
2	PB1	2341	PB1	757	Polymerase subunit; RNA elongation,endonuclease activity; interacts with PB2 and PA
			PB1-F2	87	Pro-apoptotic activity; inhibits RIG-I-dependent interferon (IFN) signaling and NOD-like receptor family pyrin-domain containing 3 (NLRP3)-mediated inflammasome formation; interacts with PB1; influences the polymerase activity
			PB1-N40	718	Maintains the balance between PB1 and PB1-F2 expression
3	PA	2233	PA	716	Polymerase subunit; protease activity/ Endonuclease activity (cap snatching); PA interacts with PB1
			PA-X	252	modulates the immune response (host inflammation)
4	HA	1778	HA	566	Surface glycoprotein; major protein; receptor binding and fusion activities
5	NP	1565	NP	498	RNA binding protein; nuclear import regulation
6	NA	1413	NA	454	Surface glycoprotein; sialidase activity, virus release
7	M	1027	M1	252	Matrix protein; vRNP interaction; RNA nuclear export regulation, viral budding
			M2	97	Ion channel; virus uncoating and assembly
8	NS	890	NS1	230	Interferon antagonist protein; regulation of host gene expression
			NS2	121	Nuclear export of vRNP to the cytoplasm
			NS3	194	Potential factor for adaptation of influenza A virus to mouse host (not yet proven)

* Length of the proteins of PB1-F2, HA, NA and NS1 varies among different subtypes/strains.

2. Avian influenza ECOLOGY AND INFECTION

2.1 Antigenic diversity and viral evolution

AIV has the ability to generate a high degree of genetic and antigenic diversity. According to the antigenic variation of the two surface glycoproteins HA and NA, currently 16 HA and 9 NA AIV subtypes are known and consequently 154 possible HA and NA combinations theoretically exist although not all have been described in nature (Hause et al., 2014a; ICTV, 2017). In contrast to this, bat IAV (H17N10 and H18N11) fail to reassort with conventional IAVs (Ciminski et al., 2017) and are therefore not included here.

The NP and M genes share a higher degree of conservation among all influenza viruses and have therefore historically been used to determine the Influenza virus type (A, B, C or D) (Yoon et al., 2014). Apart from their natural host reservoir, AIV is known to rapidly evolve in several avian hosts (Oxford J, 2003; RG. et al., 1975). Diversifying evolution of AIV is driven by three mechanisms: point mutations (genetic drift), genome segment rearrangement (reassortment; genetic shift) and RNA recombination (Shao et al., 2017).

A high rate of both synonymous and nonsynonymous point mutations is due to error-prone activity of the RdRP and the fact that there is no proof-reading mechanism for replicating negative sensed single-stranded RNA replication in eukaryotic cells (Boni, 2008; Chen et al., 2006). In case the two major surface glycoproteins of the AIV genome, the HA and/or NA gene, are affected by non-synonymous mutations, antigenic variants may result. These mutations may accumulate continually over time, and the process as such is often referred to as *antigenic drift*. Consequently, recognition by neutralizing antibodies (synthesized due to previous virus infection or vaccination) may be negatively affected (Carrat et al., 2007). This may compromise vaccination strategies as it is of utmost important to select vaccine strains that antigenically match the circulating field viruses as closely as possible. Therefore, influenza surveillance efforts are useful to detect short-term antigenic drift and, thus, aids in vaccine selection. Rates of the genetic drift of the influenza HA protein in poultry are similar to those observed in human H3 IAV that show approximately 7.9 nucleotide and 3.4 amino acid substitutions per year in the HA1 gene (Suarez et al., 2000). In contrast to this, the mutation rate of the HA1 protein of AIV H5 and H7 subtypes infected poultry species from

live bird markets in the United States showed 7.8 and 4.9 substitutions per 1000 nucleotide sites per year, respectively.

Secondly, genome segment rearrangement or *reassortment* acts as an important mechanism for genetic and antigenic diversity of influenza viruses: Simultaneous infection of a single cell with two different parental influenza viruses of different sub- and/or genotypes allows the swapping of whole gene segments during assembly of virions, thereby producing different genotypes among the progeny virions (Baigent et al., 2003; Scholtissek, 1990; Scholtissek, 1995). Hence, a new genotype can be formed if one or more of the internal gene segments are replaced and a new subtype if either HA and/or NA segments are exchanged (Steel et al., 2014). Exchanging the HA segment may lead to a complete changing of antigenic properties of the virus, hence it is referred to as *antigenic shift*. For instance, several reassortant influenza subtypes have been documented during the last decade that harbor one or more of the internal gene segments of subtype H9N2 viruses (e.g. H5N1 and H10N8 (Chen et al., 2014; Monne et al., 2013) or LPAIV H7N9 in China (Lam et al., 2013)). A pandemic may occur if a new reassortant virus (i) carrying an antigenically altered HA segment and (ii) readily transmissible, is introduced into an immunologically naïve global human population (McDonald et al., 2016; Webster et al., 1992a). Historically, AIV gene segments were contributing to the generation of human pandemic viruses that emerged in Spain in 1918-1919 (H1N1), in Asia in 1957 (H2N2), in Hong Kong in 1968 (H3N2) and in 2009 as the so-called "swine flu" in Mesoamerica (H1N1) (Garten et al., 2009; Morens et al., 2010; Shao et al., 2017). All four pandemics had their origin linked to reassortant influenza viruses of either human and avian or swine (in 2009) origin (Kilbourne, 2006; Taubenberger et al., 2009) and together cost the lives of an estimated more than 50 million human beings (Baigent et al., 2003; Johnson et al., 2002; LaRussa, 2011; Webster et al., 1992b). Due to the highly zoonotic character of some AIV, a continuing potential threat to human health is given and an influenza pandemic still remains one of the most serious threats to public health.

As a third though rare mechanism *recombination* has been described to contribute to AIV divergence. During recombination, an insertion or shuffling of viral sequences of other than HA genome segments into the HA genome segment has been reported, resulting in the emergence of HPAIV in poultry (Dhingra et al., 2018; Holmes, 2003; Maurer-Stroh et al.,

2013). For instance, HPAIV H7N3 in Chile (2002) and Canada (2004) appear to have arisen as a result of recombination with the viral NP and M genes, respectively (Pasick et al., 2005; Suarez et al., 2004).

2.2 Avian influenza virus in a global persepective

AI was first described as a clinical entity in poultry in Italy in 1878 (Perroncito, 1878) and thereafter defined as “bird/fowl plague” by the beginning of the 20th century. AIV are responsible for huge economic losses in the poultry industries nowadays (especially in Asia) and some strains pose a health risk for human beings. Based on their pathogenicity in chickens *in vivo* and the composition of the endoproteolytic cleavage site (CS, also referred to as HACS) of the HA protein a classification into two pathotypes is achieved (OIE, 2017). While the majority of AIV is of the LP pathotype, only viruses of subtypes H5 and H7 have the potential to emerge to HP variants in nature.

2.2.1 Low pathogenic avian influenza virus

LPAIVs mainly run an asymptomatic course of infection in reservoir hosts (aquatic wild birds) and induce only mild or even no obvious clinical signs in gallinaecous poultry. Due to their widespread occurrence in wild bird populations, they are sporadically transmitted to poultry populations and rarely also to mammals, including humans. LPAIV infections of H7 and H9 subtypes are the most frequently detected ones in domestic poultry, causing sublinical infections (Fusaro et al., 2011; Sun et al., 2015) which however may progress to clinical disease given presence of opportunistic co-pathogens (e.g. *E. coli, Ornithobacterium rhinotracheale* etc.; (Hassan et al., 2017; Samy et al., 2018)) and/or adverse environmental conditions (e.g. high ambient temperatures, (Harder et al., 2016)). LPAIV of subtype H9N2 continue to circulate and are actually the most prevalent subtype in poultry worldwide. There are distinct lineages endemic in several Asian and Middle-East countries affecting all poultry sectors, including commercial farms, backyards and live bird markets (Capua et al., 2007; Fusaro et al., 2011). Moreover, some H7 virus strains such as H7N2, H7N3, and H7N7 have occurred sporadically in several countries in Europe, Asia and America (Abdelwhab et al., 2014b). LPAIV of subtype H7N9 of Chinese origin shows an increased zoonotic potential (although LPAIV). Since 2013, five epidemic waves of this virus have been reported, mainly

affecting China and involving over 1500 human cases and more than 600 deaths (FAO, 2018; Su et al., 2017). Although no human-to-human transmissions have been reported so far (Bui et al., 2016), the continuous evolution of this virus represents an ongoing long-term threat to public health and the poultry industry.

Some AIV subtypes have a predilection for a narrow spectrum of avian host species (e.g. the "gull lineages" of H13 and H16 for *Charadriiformes*; (Brown et al., 2012; Munster et al., 2007) or vary according to time and location (e.g. H14 and H15 which seem to be confined to southern Siberia). Subtypes H14 and H15 have only been detected rarely even in large surveillance studies, (Kawaoka et al., 1990; Rohm et al., 1996) which leads to the suggestion that the host reservoir of these viruses may be infrequently sampled in "common studies". Within their natural reservoir, subtypes H3, H4, H5, H6 and H11 are most frequently reported from migratory birds (Kuiken, 2013; Latorre-Margalef et al., 2014; Munster et al., 2007; Olsen et al., 2006). Sporadically, some of these subtypes are transmitted to poultry and are detected in backyard flocks, commercial poultry and life-poultry markets (Lee et al., 2010; Negovetich et al., 2011).

2.2.2 Highly pathogenic avian influenza viruses

HPAIVs cause severe clinical signs, leading to high mortality rates of up 100 % especially in gallinaecous species. By definition, HP pathotypes show at least two characteristic features: An intravenous pathogenicity index (IVPI) of >1.2 and a multibasic cleavage site (MBCS) within the HA protein (Anonymous, 2005; OIE, 2017). The phenomenon of the emergence of HPAIV appears to be associated with the adaption of LPAIV precursors to domestic poultry, mainly to chickens or turkeys (Richard et al., 2017). While the majority of AIV remains LP, only viruses of the subtypes H5 and H7 have the potential (under natural conditions) to evolve from LP to HP variants (Figure 3) (Bonfanti et al., 2014; Capua et al., 2007). Nevertheless, a few viruses of subtypes H10N4 and H10N5 have been considered as HP by IVPI, although they do not harbour a MBCS and do not cause severe clinical disease when inoculated occulo-nasally (Wood et al., 1996). In addition to this, four AIV of subtype H5 have been described that express a MBCS but did not show the HP phenotype *in vivo* (IVPI of <1.2) (Londt et al., 2007). A total of 26 epizootics caused by HPAI of either subtype H5 or H7 were documented between 1959-2008 worldwide (Alexander et al., 2009; Swayne, unkown).

In 1996, the goose/Guangdong (gs/GD) H5N1-lineage emerged in Southeast Asia and was first detected at a commercial geese farm in China, Guangdong province. Over time, the HPAIV H5N1 of the gs/GD-lineage and its resassortant derivates (H5N2, H5N6, H5N5 and H5N8) spread widely and affected Europe, Africa and the Middle East, (Neumann, 2015; Wong et al., 2015; Wu et al., 2015). Some strains of this lineage even established endemic status in several Asian, Middle East and African countries including China, Vietnam, Indonesia and Egypt (Hagag et al., 2015; Kim, 2018) and repeatedly caused epizootics in Europe and North America (Guan et al., 2009). So far, the virus continues to evolve and has differentiated into ten major clades (clade 0-9) and further differentiated subclades, based on phylogenetic analysis of the HA gene (Smith et al., 2015). Three “major clades” are acutally circulating: clade 2.2.1.2 (in Egypt), clade 2.3.2.1 (mostly in Africa, Asia and Europe) and clade 2.3.4 (in Asia, Europe and North America). In additon to this, three “minor clades” (clade 1.1.2, 2.1.3.2 and 7.2) revealed an endemic status in poultry of several Asian countries. Transcontinental spread of gs/GD viruses had been linked to migratory wild birds (Capua et al., 2007; Chen et al., 2005; Viruses, 2016). Recently, outbreak waves of a new reassortant gs/GD HPAIV of subtype H5N8 occurred in Europe, Middle-East, Asia and Africa in 2016-2017 due to incursions via migratory wild birds (ECDC, 2016; Globig et al., 2017; Pasick et al., 2015; Pohlmann et al., 2017). Speed and extent of spread of the gs/GD-lineage is unprecendented to date.

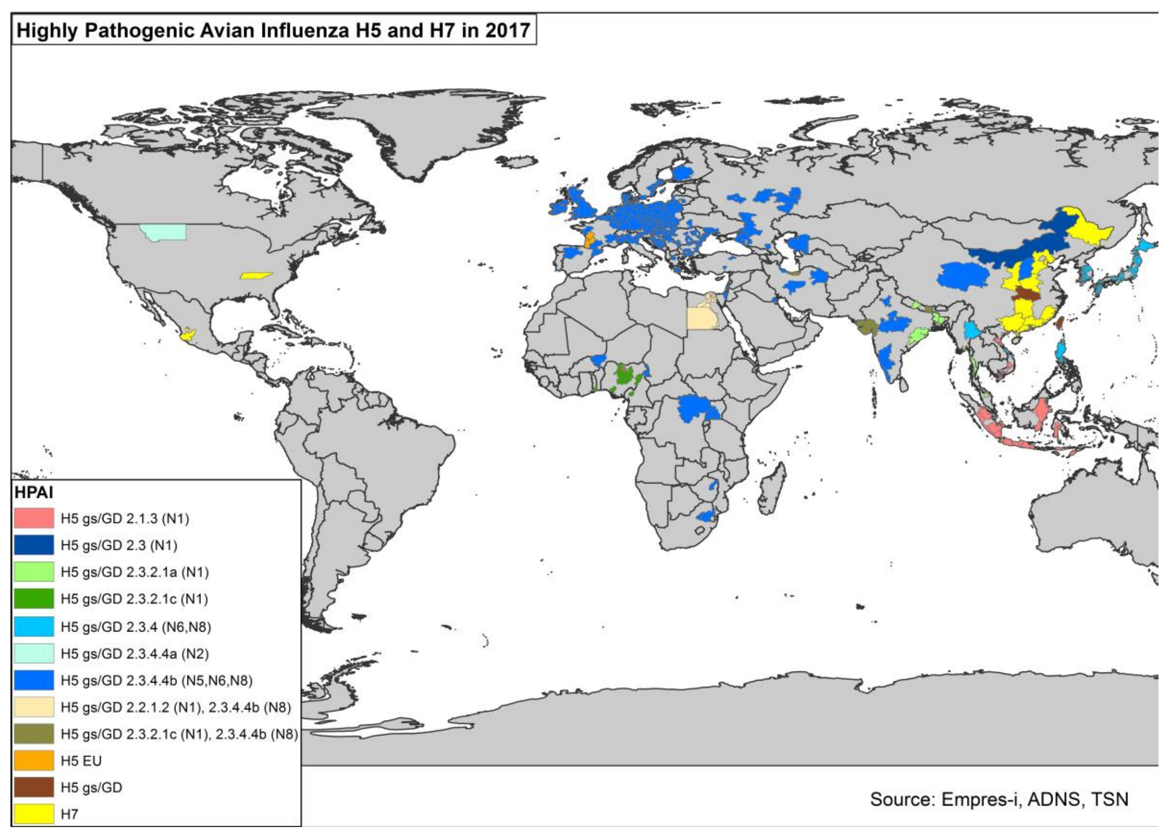

Figure 3. Geographic distribution of highly pathogenic avian influenza H5 and H7 outbreaks among poultry and wild birds in 2017 (EFSA et al., 2017).

2.3 Avian influenza virus in Europe

Over the past decades, the impact of LPAIV and HPAIV on the poultry industry in Europe has increased both in terms of numbers of infected birds and costs of applied control measures (WHO, 2018c). A drastic restructuring of industrial poultry production since the mid 1990 from small flocks into huge production units is believed to have contributed to this process (Kalthoff et al., 2010). The primary introduction of AI viruses into a poultry population often takes place as a result of direct or indirect contact to endemically infected wild bird populations (Capua et al., 2007). Therefore, the prevention of AIV incursions depends mainly on enforcement of biosecurity measures in poultry holdings (e.g. segregation, cleaning and disinfection). Europe has experienced several incursions by viruses of the H5-gs/GD lineage in both wild birds and poultry over the past decade. Contrary to several Asian countries and Egypt, no spillover infection of humans was reported from Europe until now although some of the gs/GD viruses in Europe showed zoonotic potential (Adlhoch et al., 2014).

Nevertheless, continous co-circulation in poultry in endemically infected regions in Asia and sporadic spillover into migratory wild bird populations of different HPAIV gs/GD H5 lineages poses constant risks of new incursions into Europe by migrating wild birds or in associaton with (illegal) poultry trading practices (Adlhoch et al., 2014).

Eurasian-origin LPAI H5 viruses (unrelated to the gs/GD lineage) are routinely detected in aquatic wild bird species in Europe with a higher incidence during the autumn migration period (Munster et al., 2007). Each spillover infection of LPAIV of subtype H5 and H7 into poultry bears the risk of a *de novo* generation of HP phenotypes (Franca et al., 2014). A recent example of such emergence are the HPAIV H5 N1/N3/N9 outbreaks in France that were unrelated to the concurrent gs/GD epizootics of HPAIV H5N8 (clade 2.3.4.4) that affected Europe in 2016-2017 (Guinat et al., 2018; Napp S et al., 2017). In addition to subtype H5, LPAIV of subtype H9N2 affected domestic ducks, chickens and turkeys in several countries in Europe since the mid-1990s, for instance Germany and Poland in 2013/2014 (Swieton et al., 2018). In addition to this, annual presence of LPAIV H7 subtypes in Eurasian wild bird populations as well as sporadic HPAIV H7 outbreaks have been detected and are presented in chapter 2.4.

2.4 Avian influenza virus H7 in Europe (low and highly pathogenic)

Several incursions of LPAIV subtype H7 into poultry as well as the *de novo* generation and spread of HPAIV subtype H7 have been reported from Europe over the past two decades (Table 2; (Abdelwhab et al., 2014b; Dietze et al., 2018; OIE, 2018a). Due to the high mortality rate of HPAIV in domestic poultry (Richard et al., 2017), huge economic losses of the poultry industry may ensue. The HPAIV H7 outbreak in Italy in 1999-2000 is the best known example that resulted in the death of 14 million domestic birds (Capua et al., 2000b). Moreover, HPAIV H7N7 occurred in the Netherlands, Belgium and Germany in 2003 and led to the death and culling of around 30 million chickens as well as to 89 confirmed cases of human infection and one fatal case in the Netherlands (Fouchier et al., 2004; Jonges et al., 2011; Koopmans et al., 2004). HPAIV H7 outbreaks further occurred in the UK in 2008 and 2015 (Defra, 2008, 2015; Seekings et al., 2018), in 2009/2010 in Spain (Iglesias et al., 2010), in Italy in 2013 (Bonfanti et al., 2014) and in Germany in 2015 (Dietze et al., 2018). In very

few HPAIV outbreaks, also the LPAIV precursor has been identified (Table 2). These comprise the H7N1 outbreak in Italy in 1999-2000 (Bonfanti et al., 2014; Monne et al., 2014), the H7N7 outbreak in the UK (Defra, 2008, 2015), H7N7 in Spain 2009/2010 (Iglesias et al., 2010) and H7N7 in Germany in 2015 (Dietze et al., 2018). Risks of new incursions into the poultry population in Europe are perpetuating due to the annual presence of LPAIV subtype H7 in Eurasian wild bird populations (Abdelwhab et al., 2014b).

Table 2. Outbreaks in poultry of subtype H7 avian influenza viruses of low (LP) and high (HP) pathogenicity in Europe, 1979–2016 (modified from (Abdelwhab et al., 2014b; Graaf et al., 2017; OIE, 2018a; Richard et al., 2017).

Subtype	Country	Wild birds, year*	Domestic poultry, year*
H7N1	Belgium	2008, 2009	
	Denmark	2007, 2008, 2009	2008, 2010
	France	2001	
	Germany	2004	
	Italy	2002	**1999-2000**, 2001, 2008
	Netherlands	2007	2011
	Spain	2006-2009	
H7N2	Denmark	2009	
	Italy	1993	
	Netherlands	2006	
	Spain	2009	
	United Kingdom		2007
H7N3	Germany		2008
	Italy	2001	2002-2003
	Netherlands	2000	
	Portugal	2005-2009	
	Ukraine	2010-2011	
	United Kingdom		2006
H7N4	Italy	2004-2006	
H7N6	Ukraine	2010-2011	
H7N7	Belgium	2010	**2003**
	Bulgaria	2008	
	Czech Republic	2011	
	Denmark		2013, 2016
	Germany	2005-2006	2001, **2003**, 2009, 2011, 2013, **2015**
	Hungary	2007	
	Ireland		1998
	Italy	2004, 2005	1999-2000, 2013, 2016
	Netherland		2003, 2006, 2011, 2012, 2013, 2015
	Northern Ireland		1998
	Poland	2008, 2009	
	Portugal	2005-2009	
	Slovenia	2009	
	Spain		**2009-2010**
	Sweden	2002, 2003, 2005, 2008	
	United Kingdom		**2008, 2015**
	Ukraine	2010-2011	
H7N8	Netherlands	2006	
	Spain	2004	
	Sweden	2004	
	Ukraine	2006	
H7N9	Czech Republic	2009	
	Spain	2005, 2008	
	Sweden	2002	
H7Nx	Denmark	2007-2010	
	Portugal	2005-2009	
	Switzerland	2006-2009	

* Outbreaks in poultry caused by HPAIV appear in bold face and human infections are highlighted in blue.

2.5 Avian influenza - Clinical disease in avian species

The ability of AIV to cause disease is influenced by several factors like the subtype of the virus, the dose of the inoculum, the host species, the age and genetic lineage of the bird, concurrent co-infections and environmental conditions (Alexander, 2000; Blohm et al., 2016; Umar et al., 2017; Werner, 2006). Aquatic birds, especially dabbling ducks (e.g. mallards - *Anas platyrhynchos*) represent the natural host reservoir for AIV (Causey et al., 2008; Jourdain et al., 2010). However, also other avian species and even mammals can acquire AIV infections (Freidl et al., 2014; Imai et al., 2013; Kalthoff et al., 2010; Parrish et al., 2015). According to their pathogenicity in gallinaceous poultry (e.g. chickens and turkeys), AIV can be distinguished into LP and HP. Usually, LPAIV cause mild and primarily respiratory signs that are often not associated with severe losses in poultry (Elbers et al., 2005). Depending on aggravating factors as mentioned above, reduction in weight gain in broiler chickens or a temporary decrease in egg production in layer chickens are most notable. Mortality can reach, in some rarer cases, up to 30% (Halvorson, 2008; Spackman et al., 2003).

In contrast to this, HPAIV infection in avian species, mainly in gallinaceous poultry, causes systemic disease leading to multiple organ dysfunction and death. HPAIV leads up to 100% morbidity and mortality within 36-48 hours in highly vulnerable gallinaceous species like chickens and turkeys (Alexander, 2000; Swayne et al., 2000). Infected birds rapidly develop severe clinical signs like cyanosis of the comb, wattles and legs, oedema, diarrhea, severe depression and various neurological signs (Anonymous, 2015). In contrast to chickens or turkeys, waterfowl show a protracted course of infection, ranging from nearly no clinical signs to benign enteric disease (Capua et al., 2001; Perkins et al., 2003; Shortridge et al., 1998). However, few outbreaks are known so far in which ducks developed severe clinical signs including neurological symptoms and high mortality. This includes an HPAIV H7N1 outbreak in Italy in 1999-2000 where muscovy ducks were severely affected (Capua et al., 2002), an anseriform species that seems to be more vulnerable in general compared to mallards or Pekin ducks. In addition, some strains of HPAIV gs/GD H5N1 have caused high morbidity and mortality rates in domestic and wild ducks since 2002 (Londt et al., 2008; Pantin-Jackwood et al., 2007; Sturm-Ramirez et al., 2004), and recent HPAIV H5N8 virus strains of clade 2.3.4.4b have proven lethal in several species of dabbling and diving ducks (Grund et al., 2018; van den Brand et al., 2018).

2.6 Avian Influenza virus as a zoonotic agent

The actual number of documented AIV cases in humans, although steadily growing over time, must be still considered as being comparatively low and as rare events (WHO, 2018c). Nevertheless, the potential threat to public health and society remains high due to the inherent capacity of AIV to donate by reassortment genome segments to human pandemic viruses (Freidl et al., 2014; To et al., 2012; Wei et al., 2013). In most documented cases, human infections - including fatal cases - have a history of exposure to infected poultry by either direct or indirect contact (Peiris et al., 2007). A higher zoonotic potential has been reported for the HPAIV gs/GD H5-lineage and for LPAIV/HPAIV H7N9 (Lai et al., 2016; Perkins et al., 2003; WHO, 2018c).

An important determinant of host range, and hence of zoonotic propensity, is the receptor binding site (RBS) of the HA protein that is critical for cellular receptor specificity (Gambaryan et al., 2003; Mair et al., 2014; Wilks et al., 2012). IAV bind to terminal SA of glycan structures on the host cell surface to initiate infection (Byrd-Leotis et al., 2017). Based on their chemical composition (N-acetylneuraminic acid or N-glyconeuraminic acid) and the type of glycosidic linkage to the α2-carbon of the subterminal galactose residue, by α2,3 or α2,6 connectivity, species-specific affinity varies. HA of avian viruses mainly bind to α2,3-linked SA, while the α2,6-SA is the predominant receptor in many mammalian species, including humans (Connor et al., 1994; Ito et al., 1997). Host species show different expression patterns of these two receptors: α2,3-linked SA is present in duck intestinal epithelium (Ito, 2000), while the upper respiratory tract epithelium of humans expresses mostly α2,6-linked SA (Baum et al., 1990). However, both avian and mammalian/human cells may express (often in a tissue-specific manner) SA of both linkage types, hence there is no absolute HA specificity (Wilks et al., 2012). For instance, the α2,3 SA is also present on ciliated cells of the human lower respiratory tract, and these cells can be infected with and are permissive for AIV (Matrosovich et al., 1999; Matrosovich et al., 2004a, b; Shinya et al., 2006). Consequently, extensive exposure of cells in the human lower respiratory tract might initiate interspecies infection (Peiris et al., 2007). Several amino acid sites in the HA RBS govern receptor specificity: Substitutions Q226L and G228S (H3 numbering) in the HA protein alter the host receptor binding specificity from α2,3 (avian= Q226, G228) to α2,6 (human=L226, S228) SA receptors in HPAIV H5 viruses of the gs/GD-lineage (Connor et al.,

1994; Gambaryan et al., 2012).

According to the World Health Organization (WHO), various clades of gs/GD HPAIV H5N1 have been associated with more than 860 human cases of which 454 were fatal (WHO, 2018b). These infections included only a few family clusters of human-to-human transmission but generally these viruses are not yet adapted to efficacious transmission between human hosts. However, several amino acid substitutions located near the RBS or affecting pH stability of the protein correlated with a shift in affinity of the HA skewing it toward a more affine binding of the human SA receptor type (Herfst et al., 2012; Imai et al., 2012; Watanabe et al., 2011; Zhao et al., 2013). In addition, HPAIV H5N6 clade 2.3.4.4c recorded 19 confirmed human cases including 6 deaths in China since 2014 (WHO, 2018a).

Chinese LPAIV subtype H7N9 raised public health concern as the only LPAIV that has caused severe human disease and fatalities since February 2013 due to the presence of the Q226L substitution in the HA gene (1625 confirmed human cases and 623 deaths) (FAO, 2018). However, no human-to-human transmission has been observed for these viruses so far (Bui et al., 2016).

Some other H7 subtypes express zoonotic properties, and several sporadic human infections have been reported since 2002 (Belser et al., 2009; Belser et al., 2007), Table 2). Affected humans usually showed only minor clinical signs like conjunctivitis, sneezing or coughing (Anonymous, 2007; Nguyen-Van-Tam, 2006a; Nguyen-Van-Tam, 2006b; Puzelli et al., 2005, 2006). During an HP H7N7 epizootic in the Netherlands in 2003, mild conjunctival infections in 88 human cases and one severe fatal case were recorded (Elbers et al., 2004; Fouchier et al., 2004). Another HPAIV H7N7 outbreak in Italy in 2013 resulted in infections of three poultry workers and farmers presenting with conjunctivitis and influenza-like-illness (Puzelli et al., 2016; Puzelli et al., 2014). Both, HPAIV H7N7 in humans of the Dutch and Italian outbreak, revealed a substitution at position 143 of the HA gene (A143T) known to introduce a potential glycosylation site at position 141 (near to the RBS) that may affect the receptor binding specificity or affinity of the HA (de Wit et al., 2010b).

In addition, LPAIV subtype H9N2 of the G1 lineage have caused 31 human infections in several Asian countries; these viruses carry HA aa substitutions H191 and L234 (H9 numbering) that enable binding of a wider range of SA receptors including human ones (Sun

et al., 2015; Wan et al., 2007). Moreover, LPAIV H9N2 in Egypt that also acquired human-like α2-6 SA receptor specificity (Matrosovich et al., 2001), provoked four human cases with only mild symptoms and no evidence of human-to-human transmission until now (Flutrackers, 2017; WHO, 2018c). In December 2013, a severe disease was triggered by LPAIV subtype H10N8 in China where two out of three infected humans died (Chen et al., 2014; Liu et al., 2015). There is actually only a single human case of H6N1 infection that took a benign course (Yan et al., 2014).

3. Avian influenza PATHOGENESIS

3.1 Definition of avian influenza pathogenicity

Based on the pathogenicity in chickens, AIV has been distinguished as phenotypes of low and high pathogenicity. The World Organization for Animal Health (OIE) established criteria for the pathotype identification based on (i) the nucleotide sequence of the HA cleavage site (CS) and (ii) *in vivo* testing in either eight four-to-eight week-old or 10 six-week-old chickens (IVPI) (OIE, 2015b). Therefore, any H5 or H7 virus with a multibasic HACS, or any AIV that expresses an IVPI of >1.2 (equivalent to at least 75% mortality of four-to-eight-week old chickens within 10 days after intravenous inoculation) is considered to be of the HP phenotype. All other viruses are referred to as LPAIV.

3.2 Molecular basis of pathogenicity

Determining the AIV pathotype is mandatory for any legal binding diagnosis during surveillance, investigations of suspect cases and establishment of control measures of notifiable AIV infections. Molecular pathotyping by nucleotide sequencing of the HACS has been routinely established in order to save time to diagnosis and avoid animal experiments. The activation of HA by endoproteolytic cleavage into two subunits (HA1 and HA2) is indispensable for AIV infectivity (Klenk et al., 1975), and differences in pathogenicity of AIV subtypes H5 and H7 are mainly due to structural variations at the HACS (Chen et al., 1998). While LPAIV harbor a monobasic CS that is also referred to as single-basic cleavage site (SBCS) with a single basic amino acid (aa) (either arginine “R” or lysine “K”) at position -3 or

-4, HPAIV possess multibasic CS motifs with more than one R and/or K at least at positions -2 and -3, therefore considered as multi-basic CS (MBCS) (Figure3). An SBCS is processed by extracellular trypsin-like enzymes that are limited to the respiratory and intestinal tract (Bottcher-Friebertshauser et al., 2014). Due to the specific tissue distribution of these proteases, the spread of LPAIV infection in avian and mammalian hosts is limited. MBCSs are processed by subtilisin-like enzymes like furin which are ubiquitous in all tissues (Garten et al., 2008a); viruses expressing an MBCS therefore are capable of systemic infection. Nevertheless, the MBCS is apparently not always the sole feature that defines the HP phenotype (Abolnik et al., 2009; Londt et al., 2007; Stech et al., 2009; Veits et al., 2012). Thus, shifting of pathogenicity is not fully limited to the molecular composition of the HACS but further, as yet ill-defined, mechanisms and/or other gene segments may be also involved (Abdelwhab et al., 2013).

A list of HACS motifs obtained from outbreaks around the world has been published by the “OIE FAO network of expertise on animal influenza” (OFFLU et al., 2018) that lists variations in the sequence and critical number of basic aa and represents a helpful tool in defining molecular HP criteria. An isolate should be considered as HP pathotype, if the aa motif is similar to at least one of the HP isolates in that list.

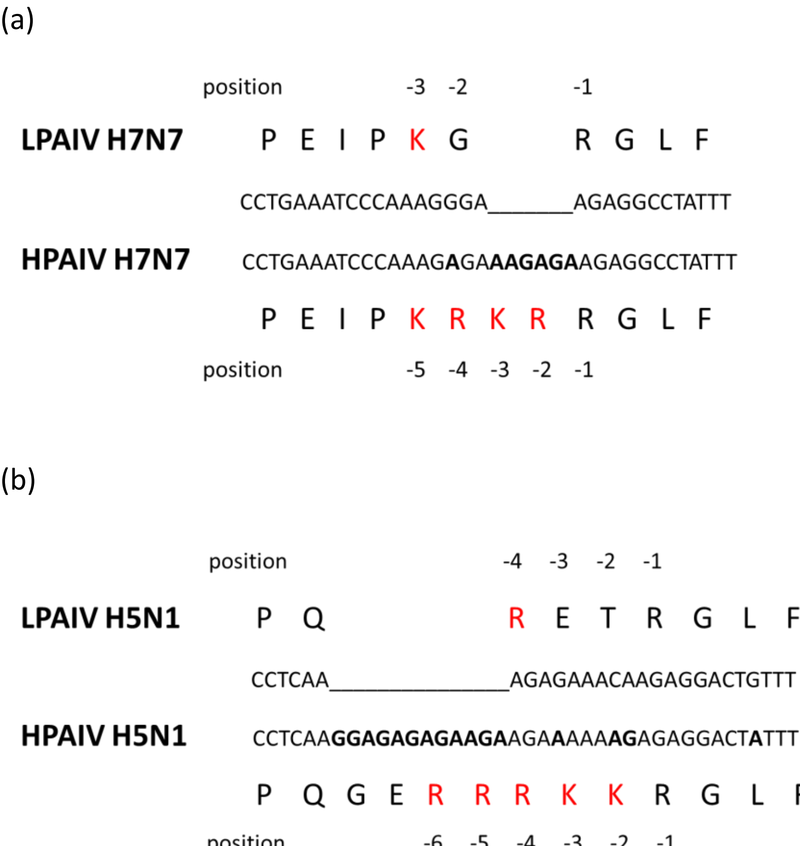

Figure 4a-b. Hemagglutinin cleavage sites (HACS) of LP and HPAIV subtypes H7 and H5, respectively. The figures show the HACS of an LPAIV isolate with a single-basic HACS with a single basic residue and a corresponding HPAIV isolate with more than one basic residue. Basic amino acids residues K (lysine) or R (arginine) present at the HACS (excluding position -1) are highlighted in red. Figure (a) shows the pair of LP/HP H7N7 viruses (deposited in the EpiFlu^{TM} database (platform.gisaid.org) under accession numbers EPI_ISL_191763-64 and EPI_ISL_191941-42) that has been detected in Germany in 2015. In Figure (b) the HACS of a typically Eurasian wild bird LPAIV subtype H5 is shown (accession number CY107849) and the MBCS of a gs/GD descending HPAIV H5 of clade 2.3.4.4a (accession number AM408215.1).

3.3 Mutational switch from low to high pathogenicity

The exact molecular mechanisms that favor a mutation from LP to HPAIV are still unknown. Conceivable mechanisms for the emergence of HPAIV from LP precursors by converting a single-basic to a multibasic HACS have been proposed based on outbreaks that have occured in nature (Table 3). The three commonly observed “major mechanisms” are (i) substitution,

(ii) insertion and (iii) recombination (Perdue et al., 2003).

Nucleotide substitution refers to the mechanism, during which one or more bases of a codon in the HACS are mutated non-synonymously leading to the coding of an additional R or K. Until now, this mechanism has only been observed in association with the insertion of nucleotides (mechanism ii). HPAIV outbreaks that included base substitutions have been described in Mexico in 1994-1995 (H5N2; (Garcia et al., 1996; Horimoto et al., 1995b; Perdue et al., 1997), in Pakistan in 1995/1995 (H7N3; (Abbas et al., 2010) and in the UK in 2008 and 2015 (H7N7; (Defra, 2008, 2015; Seekings et al., 2018).

Insertion (ii) of a series of untemplated A and G residues encoding basic aa at the HACS as a result of repeated reading and duplication of an existing template by a polymerase stuttering mechanism seems to be the most frequent mechanism (Pasick et al., 2005). For instance, this mechanism has been proposed at the basis of outbreaks in chickens of HPAIV H7N7 in Australia in 1976 (Bashiruddin et al., 1992), Spain in 2009/2010 (Iglesias et al., 2010), Germany in 2015 (Dietze et al., 2018) and recently in turkeys in the United States, Indiana, 2016 (H7N8; (Killian et al., 2016), in Mexico in 1994-1995 (H5N2), in Pakistan in 1995 and 2003 (H7N3), and in England 2008 and 2015 (H7N7).

Rarely observed non-homologous recombination (iii) results in the insertion of nucleotides from another viral gene segment or other non-viral (host) sources into the HACS. For instance, the HACS of an H7N1 LPAIV from Italy mutated into unique HPAIV HACS motifs in 1999-2000 (PEIPKGSRMRR*GLF or PEIPKRSRVRR*GLF, respectively) by insertion of four aa (underlined) likely by a recombination step from an unknown source (Banks et al., 2001; Capua et al., 2002; Monne et al., 2014). As further experiments with these viruses showed, the MBCS was not sufficient for conversion to high virulence alone; accordingly, further virulence markers i.e. three aa substitutions in the HA2 domain were found to be required for full virulence conversion in chickens (Abdelwhab et al., 2016a). In addition, the HACS of an H7N3 virus in Chile in 2002, and an H7N3 virus from Canada in 2004 harbored several nucleotides from the NP and the M gene, respectively, at the HACS (Pasick et al., 2005; Suarez et al., 2004). Two examples of recombination with chicken ribosomal RNA were seen in Canada in 2005 (H7N3, 18 nucleotides; (Berhane et al., 2009) and in Mexico in 2012 (H7N3, 24 nucleotides; (FAO, 2012; Maurer-Stroh et al., 2013).

Positive selection of minor variants of HPAIV (Domingo, 2010) from a quasispecies cloud of LP variants have been proposed as a further HPAIV emergence mechanism, for example during the H7N1 outbreak in Italy in 1999-2000 or the German HPAIV H7N7 outbreak in 2015. In both examples, next generation sequencing (NGS) technology detected minor variants of HPAIV that existed as a minor population in the quasispecies of the LPAI progenitor virus (Dietze et al., 2018; Monne et al., 2014).

Unusual in comparison to other HPAI viruses, four AIV of subtype H5 that mutated to HP variants have been described that expressed a MBCS but did not show the HP phenotype *in vivo* (IVPI of <1.2) (Londt et al., 2007), confirming that virulence markers need not be limited to the HACS. For instance, the HPAIV H5N2 strain A/chicken/Pennsylvania/1370/83 isolated during an epizootic in Pennsylvania, mutated from a LPAI precursor virus that was in circulation for months and already harbored a MBCS, even though it was of low pathogenicity in chickens. When suddenly a HP variant of this virus popped up, sequencing revealed a non-synonymous mutation at position 11 of the HA protein which resulted in the loss of a glycosylation site (Deshpande et al., 1987; Ohuchi et al., 1989). It is postulated that the glycosylation at HA11 shielded access of furin-like proteases which only became fully processible after loss of the glycosylation.

Historically, layer chickens appeared to be mainly involved in the natural genesis of HPAI variants; exceptions are two events in turkeys (H5N9 in Ontario in 1996, H7N8 in Indiana, 2016 (Table 2)). In addition to this, the majority of naturally occurring emergence events with known LPAIV precursors is of subtype H7 compared to relatively few ones of subtype H5 (Richard et al., 2017). The reason for this is still unknown but highlights that the emergence of HPAIV H7 (and H5) remains a major concern for the global poultry industry as long as LP precursor viruses are in circulation as has been demonstrated repeatedly and recently in England in 2008 and 2015, Spain in 2009, Italy 2013 and Germany in 2015, demonstrating that mutation of subtypes H7 from LPAIV to HPAIV poses a recent threat (OIE, 2018b).

In vitro, *in vivo* and *in ovo* experiments or rather a combination of these methods have been established to mimic the natural mutation of LPAIV to HPAIV in the field and to further explain the mechanisms involved in the emergence by either passaging and/or the use of

reverse genetics. The emergence of HPAIV has been successful several times *in vitro* (in chicken embryo cells and Madin Darby Canine Kidney (MDCK) cells) and *in ovo* following passaging of LPAIV H5 and H7, and resulted in the insertion of basic amino acid at the HACS (Brugh, 1988; Horimoto et al., 1995b; Khatchikian et al., 1989; Li et al., 1990; Orlich et al., 1994; Orlich et al., 1990). Further successful attempts of HPAIV generation were achieved by chicken air sac passaging, followed by cerebral passaging of LPAIV H5N2 and H5N3 in chickens (Ito et al., 2001; Soda et al., 2011b). Regarding reverse genetic techniques, several studies have shown that the furin-sensitive HACS motif plays a major, but not exclusive, role in the conversion of phenotypes, when inserting a MBCS into a LPAIV strain of either H5 or H7 subtype (Abdelwhab et al., 2016a; Bottcher-Friebertshauser et al., 2014; Gohrbandt et al., 2011; Munster et al., 2010). Some studies failed to generate HPAIV by use of this technique (Schrauwen et al., 2011; Stech et al., 2009) indicating that further, as yet undefined, arrangements (in addition to the insertion of a MBCS) in either the HA and/or other gene segments of the AIV are required (Abdelwhab et al., 2013; Diederich et al., 2015; Soda et al., 2011b).

Table 3. Naturally occurring cases of HPAIV emergence from known LP precursor viruses (modified from Seekings (2017)).

Subtype	Country	Year	Species	Proposed mechanism
H5N2	Pennsylvania	1983	chicken	Deglycosylation by a T13K mutation in the HA protein
	Mexico	1994-1995	chicken	Insertion, base substitution
H5N9	Ontario	1966	turkey	Presumed reassortment with H5N1
H7N1	**Italy**	1999-2000	turkeys; chickens; other	Recombination; minor variants in the LP progenitor quasispecies
H7N3	Pakistan	1994-1995	chicken	Base substitution and interstion
	Chile	2002	chicken	Non-homologous recombination with NP
	Canada	2004	chicken	Recombination with M1, base substitution mutation
	Canada	2007	chicken	Recombination with host RNA
	Mexico	2012	chicken	Recombination with host 28s rRNA
H7N7	Australia	1976	duck; chicken	Duplication insertion
	Netherlands	2003	chicken	presumed reassortment with H7N3 2003 and H10N7 2000
	United Kingdom	2008	chicken	Base substitution and interstion
	Spain	2009-2010	chicken	Insertion of foreign nucleotides
	United Kingdom	2015	chicken	Base substitution and interstion
	Germany	2015	chicken	Substitution and insertion; minor variants in the LP progenitor quasispecies
H7N8	Indiana	2016	turkey	Insertion

CHAPTER III: STUDY OBJECTIVES

III. STUDY OBJECTIVES

Although several studies already support the concept of HPAIV emergence from LPAIV precursors after transmission of subtype H5 and H7 AI viruses from wild birds to poultry, the present knowledge of the factors governing the mutation event and the spread of de novo generated HPAIV remains patchy. This project defined three objectives to improve the understanding of these processes:

1. Complementing the AI diagnostic algorithm by developing rapid, sequencing-independent pathotyping assays based on real-time RT-PCRs (RT-qPCR) (chapters 4.1 and 4.2, pp. 39-52 and 53-66)**.**

Risks of new incursions of LPAIV and HPAIV H5 and H7 into wild birds and poultry populations in Europe are perpetuating and justify stringent control measures. Rapid differentiation between LP and HP phenotypes of these subtypes is an essential step in the diagnosis of these notifiable infections. In order to circumvent biological pathotyping (virus isolation, animal inoculation) as well as nucleotide sequencing (molecular pathotyping) and to gain precious time, development of sensitive RT-qPCRs that allow identification and distinction of Eurasian subtype H5 and H7 by probe-assisted detection of the HACS was attempted.

2. Dissecting the LP precursor/HP relationship by molecular and epidemiological analysis of a linked LP/HPAIV H7N7 outbreak in Germany in 2015 (chapter 4.3, pp. 67-91).

Direct evidence from the field of LP to HP mutations has rarely been reported. By use of available specimens of two spatio-temporally linked outbreaks of H7N7 in layer chickens in Germany in 2015, molecular characterization enabled the identification of a potential LPAIV precursor and an HPAIV effector virus. Epidemiological and molecular factors fostering this conversion were investigated taking advantage of the tools developed under item 1 of the objectives.

3. Understanding the escape of the HP effector virus following *de novo* generation from its LP precursor by experimental co-infection studies in chickens and in embryonated chicken eggs (chapter 4.4, pp. 93-138)**.**

Determining the drivers of HPAIV emergence from LPAIV is crucial for a better understanding why and when certain LP strains pose a risk of becoming HP. There is insufficient knowledge how the two AIV pathotypes interact when simultaneously infecting poultry. *In vivo* and *in ovo* co-infection experiments were conducted using the natural pair of LPAIV and HPAIV H7N7 identified in item 2 of the objectives, involving the tools developed under item 1. The study concentrated on how a minority of mutated HP virions after *de novo* generation in a single host might individually amplify and spread in that host and become transmitted within a poultry population that experiences concurrent infection by an antigenically identical LP precursor virus.

CHAPTER IV: RESULTS

IV. RESULTS

The reference section of each manuscript/the results is presented in the style of the respective journal and is not included at the end of this document. The numeration of figures and tables corresponds to the published form of each manuscript.

4.1 PUBLICATION I

Real-time reverse transcription PCR-based sequencing-independent pathotyping of Eurasian avian influenza A viruses of subtype H7

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SHORT REPORT Open Access

Real-time reverse transcription PCR-based sequencing-independent pathotyping of Eurasian avian influenza A viruses of subtype H7

Annika Graaf, Martin Beer and Timm Harder*

Abstract

Low pathogenic avian influenza viruses (LPAIV) of the subtypes H5 and H7 are known to give rise to highly pathogenic (HP) phenotypes by spontaneous insertional mutations which convert a monobasic trypsin-sensitive endoproteolytical cleavage site (CS) within the hemagglutinin (HA) protein into a polybasic subtilisin-sensitive one. Sporadic outbreaks of notifiable LPAIV H7 infections are continuously recorded in Europe and in Asia, and some lineages showed zoonotic transmission. De novo generation of HPAIV H7 from LPAIV precursors has been reported several times over the past decade. Rapid differentiation between LP and HP H7 virus strains is required as a prerequisite to emplace appropriate control measures. Here, reverse transcription real-time PCR assays (RT-qPCR) were developed and evaluated that allow LP and HP pathotype identification and distinction by probe-assisted detection of the HACS. These new RT-qPCRs allow a sensitive and highly specific pathotype identification of Eurasian subtype H7 AIV in allantoic fluids as well as in diagnostic field samples. RT-qPCR assisted pathotyping presents a rapid and sensitive alternative to pathotyping by animal inoculation or nucleotide sequencing.

Keywords: Avian influenza, Hemagglutinin subtype H7, Pathotyping, Real-time RT-PCR, Diagnosis, Cleavage site

Background

Avian influenza viruses (AIV) are members of the family *Orthomyxoviridae*, specified as influenza virus type-A. These viruses are further classified by the serologically defined subtypes of the predominant viral surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA) [1]. Their genome is composed of single-stranded, negative-sense RNA and comprises eight genome segments which encode at least ten proteins [2]. All 16 HA and nine NA AIV subtypes can be detected in populations of aquatic wild birds which form the natural reservoir of these viruses [3].

Based on their pathogenicity in chickens, two phenotypes of AIV are distinguished: highly pathogenic (HP) AIV and AIV of low pathogenicity (LPAIV). In nature, HP phenotypes have been restricted to viruses of subtypes H5 and H7. HPAIV arises from LPAI precursor viruses by spontaneous mutations leading to the insertion of basic amino acids into the cleavage site (CS) of the hemagglutinin protein (HA) which renders the HACS processible to subtilisin-like host proteases that are ubiquitous in all host tissues. Such viruses, therefore, gain competence for fatal systemic infections in avian hosts. LPAIV, in contrast, depends on local provision of trypsin-like proteases at the epithelial surfaces of the respiratory and/or gastrointestinal tracts and per se do not cause severe clinical signs [4]. All LPAIV and HPAIV infections of subtypes H5 and H7 in poultry are notifiable to the World Organization for Animal Health (O.I.E.). [5] Determination of the type of HACS is of utmost importance for the diagnosis of these infections. This can be achieved biologically by determination of the intravenous pathogenicity index (IVPI) in experimentally inoculated chickens or molecularly by nucleotide sequence analysis of the site encoding the HACS [6]. Since animal

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Table 1 Outbreaks in poultry of subtype H7 avian influenza viruses of low (LP) and high (HP) pathogenicity in Europe, 1999–2016 [20]

Year	Country	Subtype	Pathotype	Number of infected holdings
1999–2000	Italy	H7N1	HP	1
2003	Netherland	H7N7	HP	255
2008	United Kingdom	H7N7	HP	1
2009	Germany	H7N7	LP	1
2009/2010	Spain	H7N7	LP/ HP	1/1[a]
2011	Germany	H7N7	LP	23
2013	Denmark	H7N7	LP	1
2013	Italy	H7N7	HP	6
2015	United Kingdom	H7N7	LP/ HP	1/1
2015	Germany	H7N7	LP/ HP	1/1
2015	Netherland	H7N7	LP	2
2016	Denmark	H7N7	LP	1
2016	Italy	H7N7	HP	2

[a] Slash indicates that a matching pair of LP precursor and HP mutant viruses had been detected

experiment facilities or expensive equipment are required for either pathway, solutions for alternative techniques have been sought in the past: These included restriction enzyme cleavage patterns [7], probe hybridization [8] and real time RT-PCR (RT-qPCR) approaches [9]. Based on the widespread availability of RT-qPCR technology in diagnostic laboratories and its recent favorable use in pathotyping of HPAIV H5 of the goose/Guangdong (gs/GD) lineage [10], this study was conducted to develop and validate sequencing-independent RT-qPCRs for pathotyping of Eurasian H7 AI viruses.

Over the past two decades, several incursions into poultry of subtype H7 LPAIV as well as the de novo generation and (in one case) spread of H7 HPAI viruses have been reported from Europe (Table 1). Other H7 LPAIV lineages have arisen in Eastern Asia, and one of them (H7N9/China) showed significant zoonotic propensities in annual waves of poultry-to-human transmission with more than 550 fatal human cases [11, 12]. Recently, the H7N9 lineages has also yielded an HP mutant which is spreading in southern China [13]. Considering the annual presence of LPAIV of subtype H7 in Eurasian wild bird populations [14] risks of new incursions into poultry in Europe are perpetuating.

Methods

Based on the alignments of the HA H7 gene of a comprehensive selection of sequences from LP (n = 60) and HPAIV (n = 21) of Eurasian origin collected over the last decade in sequence databases (GenBank at NCBI; EpiFlu of the Global Initiative on Sharing Avian Influenza Data (GISAID)), a set of six primers was designed (Table 2). The selected primers targeted a short fragment of the HA gene that spans the endoproteolytic CS region [15–17]. The primers were designed for the broadest possible reactivity with recent Eurasian H7 sequences.

For validation of the assays, viral RNA from reference H7 LPAIV and HPAIV was used. Moreover, non-H7 influenza subtypes H5 and H9 as well as other avian respiratory viruses (infectious bronchitis virus (IBV), Newcastle disease virus (NDV)) were tested (Table 3). Viral RNA was purified with the QIAamp®Viral RNA Mini Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions. Primers were first evaluated in conventional RT-PCRs. The PCR reactions were carried out on a CFX96 thermocycler machine (Bio-Rad) using the following temperature profile: 30 min at 50 °C (RT), 2 min at 94 °C (inactivation of reverse

Table 2 Primers and probes used for sequencing-independent pathotyping of Eurasian avian influenza A subtype H7 viruses by real time RT-PCR

Primer/Probe ID	Sequence (5′ to 3′)	Location	Amplicon size	Accession number[a]
H7_CS-F1	TGMTGCTRGCAACAGGAAT	989–1007	107[b]	KX979524
H7_CS-F2 N	TGCTACTRGCAACAGGGAT	989–1007		
H7_CS-F3	TGMTGCTGGCAACWGGRAT	968–986		
H7_CS-R1N	CGTCAATKAGRCCTTCCCA	1096–1078		
H7_CS-R2N	TCCATTTTCWATRAAACCYGC	1056–1036		
H7_CS-R3	CATCAAYCAGACCYTCCCA	1056–1076		
H7_CS-LP-FAM	C + C + AAAG + GGA + A + GAG + GC	1026–1040		KY676327.1
H7_CS-HP_EMS-FAM	CCAAAGAGAAAGAGAAGAGGCC	1027–1046	120[c]	AB438941
H7_CS-HP_IT-FAM	TTCCAAAAGGATCGCGTGTGAGGA	1004–1027		KF493066

[a]Accession number of sequence/virus used to position the oligonucleotide along the HA gene
[b]size applied to LP sequences
[c]size applied to HP sequences
+ indicates that the following position constituted a "locked" nucleotide (LNA)

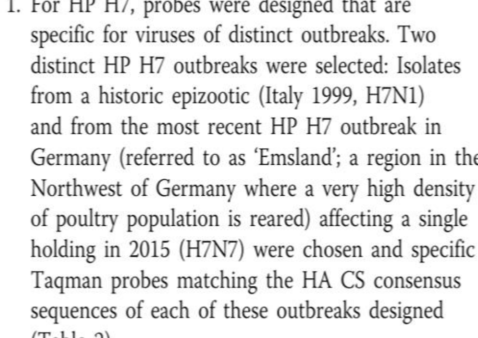
Table 3 Analytical performance characteristics of real time RT-PCR (RT-qPCR) assays for sequencing-independent pathotyping of Eurasian reference H7 viruses

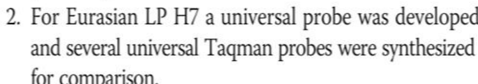
Reference virus	Accession number of HA	Sub- and pathotype	RT-qPCR method		
			LPAI H7	HPAI H7 'Emsland'	HPAI H7 'Italy'
A/mute swan/Germany/R901/2006	EPI359695	LP H7N7	Pos	Neg	Neg
A/Anhui/1/2013	AHZ60096	LP H7N9	Pos	Neg	Neg
A/chicken/Germany/AR1385/2015	SA	HP H7N7	Neg	Pos	Neg
A/broiler/Italy/445/1999	AJ580353	HP H7N1	Neg	Neg	Pos
A/turkey/Germany/R2025/2008	SA	LP H5N3	Neg	Neg	Neg
A/turkey/Germany/AR2485–86/2014	EPI552746	HP H5N8	Neg	Neg	Neg
A/chicken/Egypt/AR753–14/2013	EPI557457	HP H9N2	Neg	Neg	Neg
A/chicken/Sudan/AR251–15/2014	KX272465	IBV	Neg	Neg	Neg
A/chicken/Egypt/AR254–15/2014	SA	NDV	Neg	Neg	Neg

LP low pathogenic, *HP* high pathogenic, *Neg* negative, *Pos* positive, *SA* sequence available from the authors, also represented in the alignment in Additional file 1: Figure S1, *IBV* Infectious bronchitis virus, *NDV* Newcastle disease virus

transcriptase/activation of *Taq* polymerase), followed by 42 cycles of 30 s at 94 °C (denaturation), 30 s at 56 °C (annealing), and 30 s at 68 °C (elongation). Twenty-five μL per reaction were prepared using the SuperScript III One-Step RT-PCR system with Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA): For one reaction, 6.5 μl of RNase-free water, 12.5 μl reaction mix (2×), 1 μl of SuperScript III RT/Platinum *Taq*, and 5 μl of template RNA were mixed. Pre selected primers were then screened for their specifity using non H7-subtypes. Amplificates of the expected sizes were generated from both LP and HP phenotypes of subtype H7 viruses by conventional RT-PCR and visualized on an 2% agarose gel (Fig. 1).

Having assured the broad but exclusive specificity of the selected primers for Eurasian H7 viruses, matching probes for use in the RT-qPCR assays were developed. Initially, probes were designed with the aim to universally differentiate between LP and HP Eurasian H7 CS sequences. Probes were therefore placed directly across the sequence stretch encoding the CS. Closer inspection of the alignments, and taking into account also the list of HP H7 CS sequences provided by OFFLU [6], revealed that HP H7 CS sequences of Eurasian origin viruses were highly divergent: Viruses of separate outbreaks and epizootics represented unique CS sequences with little homology to viruses of other outbreaks. Within an outbreak series, however, HP H7 CS sequences proved to be conserved. This situation is opposed to HPAI H5 viruses of the gs/GD lineage which show considerable conservation even across different clades and allowed designing of a universal conserved probe for the HP phenotype of these viruses [10]. In contrast to HP H7, the HA CS of LP H7 viruses of Eurasian origin origin appeared to be fairly conserved [6]. Therefore, two strategies were followed to prove that sequencing-independent pathotyping by RT-qPCRs is principally possible also for Eurasian H7 viruses:


1. For HP H7, probes were designed that are specific for viruses of distinct outbreaks. Two distinct HP H7 outbreaks were selected: Isolates from a historic epizootic (Italy 1999, H7N1) and from the most recent HP H7 outbreak in Germany (referred to as 'Emsland'; a region in the Northwest of Germany where a very high density of poultry population is reared) affecting a single holding in 2015 (H7N7) were chosen and specific Taqman probes matching the HA CS consensus sequences of each of these outbreaks designed (Table 2).


2. For Eurasian LP H7 a universal probe was developed and several universal Taqman probes were synthesized for comparison.

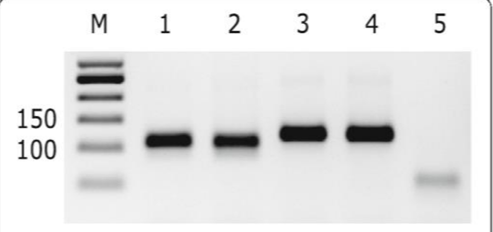


Fig. 1 PCR-products generated to distinguish between low and high pathogenic avian influenza viruses of subtype H7. Primers listed in Table 2 were used for amplification. M - DNA size marker (50 bp ladder); 1 - LP H7N7 (A/mute swan/Germany/R901/2006); 2 - LP H7N9 (A/Anhui/1/2013); 3 – HP H7N1 (A/broiler/Italy/445/1999); 4 - HP H7N1 (A/chicken/Germany/AR1385/2015); 5 - LP H5N2 (A/teal-Foehr/Wv1378–79/2003) used as negative control

The same PCR conditions as described above for conventional RT-PCR were used for RT-qPCR, however, 2 μL of the RNAse-free water were replaced by 2 μl specific primer-probe mix. The HP mixes were composed of 1,25 pmol probe/μl and 3,75 pmol/μl for each forward and reverse primer.

Results

Specificity was initially confirmed only for the two HP probes which specifically reacted with their homologous sequences but did not cross react with LP H7 or other HP H7 viruses (Table 3). The standard Taqman LP probes, however, did not sharply distinguish between pathotypes and cross reacted with various HP H7 viruses (not shown). Closer inspection of the alignments revealed that a single G/A mutation in the HA CS distinguished between LP and HP pathotypes (Fig. 2). Consequently, an LNA probe was designed placing the critical nucleotide position at the centre of the respective probe. Using this probe at a concentration of 2,5 pmol in the reaction mix finally allowed clear-cut distinction between LP and HP pathotypes by RT-qPCR (Table 3).

The detection limit of the H7 pathotyping RT-qPCRs was determined by testing ten-fold serial dilutions of viral RNAs extracted from representative H7 LPAI and HPAI viruses. Average values of three independent runs were used for comparisons to a generic RT-qPCR for the M gene of these viruses [18]. A standard curve of each assay was generated showing a linear relationship between the log dilution of the viral RNA and the cycle quantification (Cq) value for both the specific and the generic assays (Fig. 3a-c). Considering the universal LP as well as the 'Emsland'-specific HP probe, no significant difference between the median Cq values of each specific assay and the M RT-qPCR was found indicating that the newly developed and the generic RT-qPCRs have a similar analytical sensitivity. In contrast, the RT-qPCR detecting the historic Italian H7 HP lineage showed slightly higher sensitivity than the generic M RT-qPCR.

Furthermore, we determined the ability of the H7 pathotyping RTqPCRs to detect mixtures of RNAs of LPAIV and HPAIV derived from the Emsland outbreak in Germany, 2015, and compared it to the M gene-specific generic RT-qPCR (Fig. 3d). Different concentrations of LP/HPAIV-mixtures (0, 0.1%, 1%, 10%, 50% and 100% LP) were generated, and HP H7 RNA was added to 100%. Both RNA species were detected by the specific RT-qPCRs in the mixtures, and the respective Cq values reflected the concentration of the RNA species in the mixtures (Fig. 3d). H7 LP RNA was not detected in the sample containing 100% H7 HP RNA, and vice versa, once more confirming the specificity of the pathotyping RT-qPCRs (Fig. 3d). Thus, these PCRs can be used to study the generation and co-circulation of H7 HPAIV from its LPAI precursor viruses.

Assessment of the diagnostic performance characteristics of the established RT-qPCRs was carried out with a collection of H7 AIV isolates (n = 48) and H7-positive field samples (n = 27) collected between 1999 and 2016. Samples were obtained from the virus repository of the German National Reference Laboratory for Avian Influenza at the Friedrich-Loeffler-Institut, Germany, or kindly provided by the OIE Reference Laboratory for Newcastle Disease and Avian Influenza in Italy, ISZVe, Padua, the Central Veterinary Research laboratory at Dubai, United Arab Emirates, the National Centre for Foreign Animal Disease, Winnipeg, Canada and the WHO Collaborating Centre, London, United Kingdom, under the patronage of the global influenza programme (Table 4). Amplificates produced from these viral RNAs by H7-specific RT-qPCR analysis were

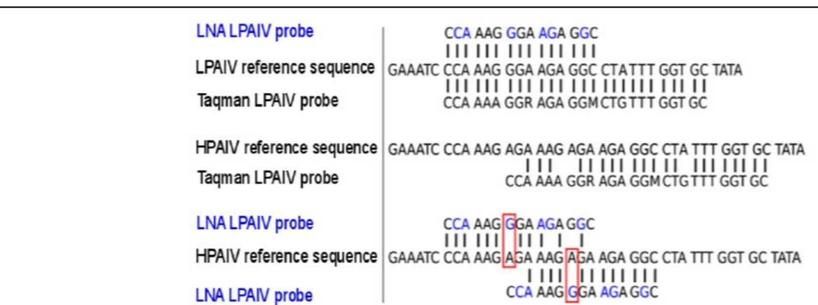


Fig. 2 Alignment of probes within the hemagglutinin gene segment site encoding the cleavage site of low pathogenic (LP, upper panel) and highly pathogenic (HP, lower two panels) Eurasian avian influenza viruses of subtype H7. Upper panel shows perfect binding of an LNA probe (LNA positions in blue color) and of a conventional Taqman probe specific for LP H7 viruses. Central panel shows the same conventional Taqman probe binding to (and cross reacting with) a Eurasian HP H7 sequence. Lower panel shows hybridization of the LNA probe to an HP H7 'Emsland' sequence; two hybridization positions are possible: The locked 'G' mismatch placed in the centre of the probe (red box) disabled binding and cross reaction at each of the two hybridization sites

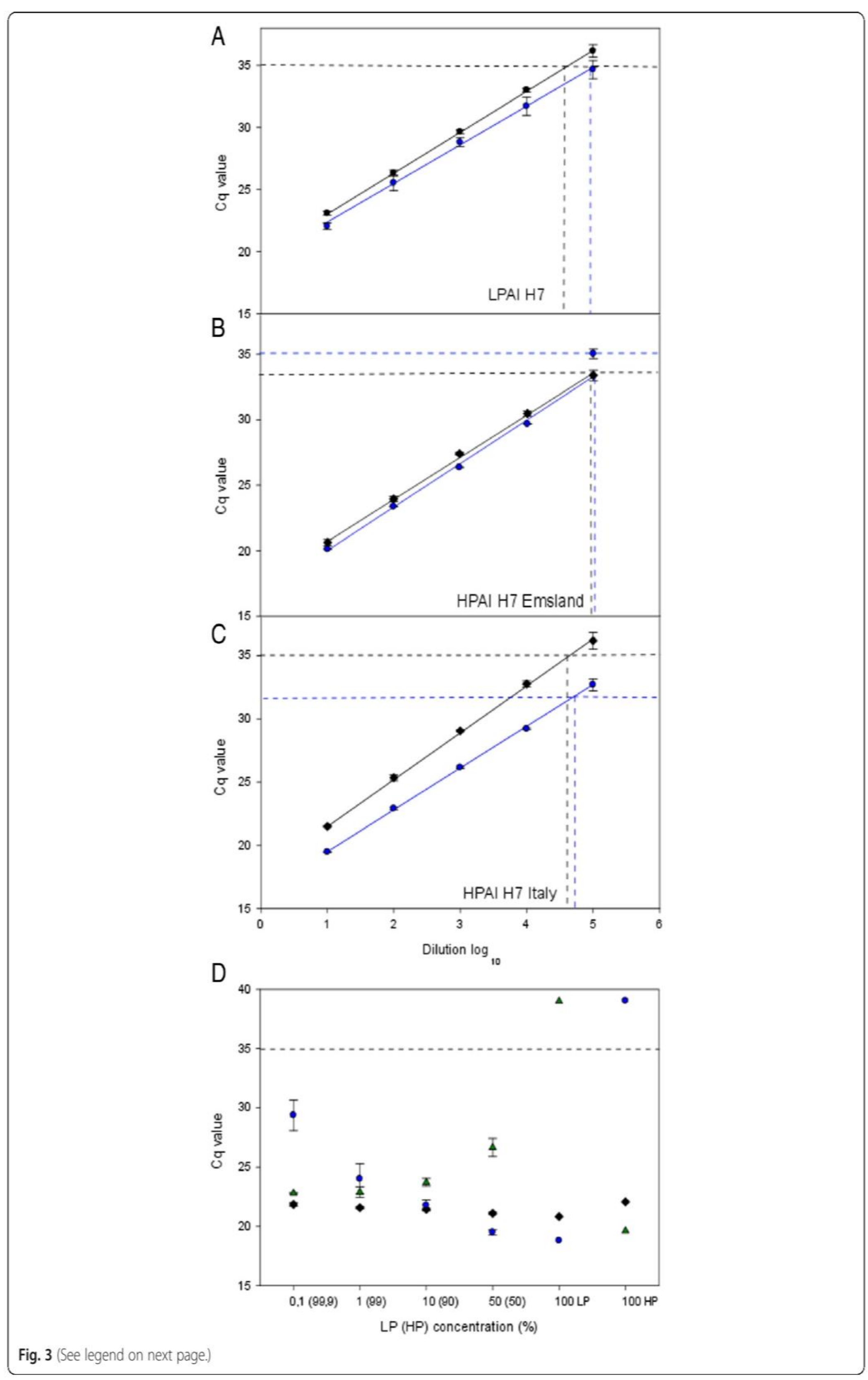


Fig. 3 (See legend on next page.)

(See figure on previous page.)

Fig. 3 Determination of the limit of detection of three newly developed RT-qPCRs for sequencing-independent pathotyping of Eurasian avian influenza H7 viruses (blue dots/lines) compared to a matrix gene-specific generic RT-qPCR (Hoffmann et al., 2010; black diamonds/lines). The detection limit was determined based on serial ten-fold dilutions using RNA of the reference viruses (**a**) A/chicken/Germany/AR1385/2015 (HPAIV H7N7), (**b**) A/mute swan/Germany/R901/2006 (LPAIV H7N7) and (**c**) A/broiler/Italy/445/1999 (HPAIV H7N1). **d** Detection of artificial mixtures of H7 LP and HPAIV RNA of the 'Emsland' outbreak compared to a matrix gene-specific generic RT-qPCR (black diamonds). RNA of the reference viruses A/chicken/Germany/AR915/2015 (LPAIV H7N7) and A/chicken/Germany/AR1385/2015 (HPAIV H7N7) were mixed and the percentage ratios indicated on the X-axis. Identification of Cq values (results of triplicate analyses) obtained for each mixture sample by H7 specific RT-qPCRs is as follows: blue circles – LPAI H7; green triangles – HPAI H7 'Emsland'

also further processed for sequence analysis using the H7-specific reverse primer mix (Table 2) for Sanger sequencing: Following agarose gel electrophoresis and amplicon purification using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) they were cycle-sequenced (BigDye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems, California, United States) and analysed on an ABI PRISM 3130 Genetic Analyzer (Life Technologies, Darmstadt, Germany) [10]. Partial HA sequences of the diagnostic samples are shown in the sequence alignment of Additional file 1: Figure S1; in all cases subtype H7 was confirmed. Pathotypes were assigned as based on the deduced amino acid sequence of the HACS according to the list of published H7 CS sequences (Additional file 1: Figure S1 and Table 4).

In total, 75 samples positive for AIV of subtype H7 were used. Based on nucleotide sequence analysis and/or IVPI, 49 samples were classified as LPAIV and 15 as HPAIV (Table 4, Additional file 1: Figure S1). They were of both historic and recent origin and mainly derived from European locations. Four samples originated from North America, nine from the United Arab Emirates/Dubai and one represented the Chinese LP H7N9 lineage. The samples mainly consisted of egg-derived isolates or native combined oropharyngeal and cloacal swabs obtained from poultry or wild birds. Seven samples were taken from the environment during a recent HPAIV outbreak in a chicken layer holding in Germany (referred to as 'Emsland'). For the H7 LP RT-qPCR, 48 out of 56 samples were correctly identified as LP (Table 4, Fig. 4), also including the Chinese LP H7N9 reference virus. Three historic LP isolates (Table 4, nos. 1–3) and the two North American LP H7 viruses (Table 4, nos. 71–72) were not detected despite high viral loads. Sequence mismatches affected binding of either probe and/or primers in these cases. In three further samples (Table 4, nos. 26, 37, 38) low virus loads were detected by the generic M RT-qPCR and these were missed by the H7 LP specific RT-qPCR. However, in most samples, the H7 LP specific RT-qPCR proved to be more sensitive as compared to the generic M specific one (Table 4, Fig. 4). Since none of the HP H7 positive samples cross reacted in the H7 LP RT-qPCR, complete specificity was achieved.

A total of 19 samples harbored HP H7 RNA. None of them was detected by the LP specific RT-qPCR (Table 4, Fig. 4). Two isolates originating from the Italian HP H7N1 epizootic of 1999 were detected by the H7 HP 'Italy'-specific RT-qPCR (Table 4, nos. 57–58); no further viruses were identified by this PCR. This includes another HPAIV H7N1 isolate from Italy originating from 2002 and distinguished from the 1999 viruses by 13 mutations in the primer and probe binding sites (Table 4, no. 56). Thus, the 'Italy 99' RT-qPCR proved to be highly lineage-specific. The second H7 HP RT-qPCR aimed at detecting HP AIV related to the most recent outbreak in Germany in 2015. All nine samples classified to harbor HP H7 were identified by this PCR with a high sensitivity (Table 4, nos. 43–51). At similarily high sensitivity four historic European HP H7 viruses (Table 4, nos. 39, 40, 42, 69), but none of the Italian HP viruses, or an isolate (Table 4, no. 41) representing the large HP H7N7 epizootic affecting the Netherlands, Belgium and Germany in 2003, reacted with either of the two HP specific RT-qPCRs. No cross reactivity to any of the LP H7 samples was detected indicating excellent performance values regarding sensitivity and specificity. Due to our results, the threshold distinguishing reliably between positive and negative samples was set at Cq = 38.

Discussion

Although not all of the LP and HP H7 samples did show a positive signal with the respective RT-qPCR due to mismatches in the probe binding regions, the newly developed set of primers produced a sequenceable amplificate even of those virus strains. Consequently, pathotype confirmation of a H7 positive sample that tested negative by the LP and HP RT-qPCRs is still possible by nucleotide sequence analysis using the amplificate produced by these RT-qPCRs. In this respect, the newly developed RT-qPCRs resemble the one introduced by Slomka et al. [19] which also spanned the H7 HACS but its probe targeted a highly conserved sequence stretch outside the CS.

Table 4 Diagnostic performance characteristics of the H7 pathotyping RT-qPCRs using HP and LP influenza A subtype H7 virus isolates and field samples collected from different countries and poultry holdings or wild bird species, 1999–2016

No.	Sample ID	Type of sample	Accession Number[a]	Subtype/ pathotype	PCR results (cq value)			
					M1.2	LP H7	HP H7 Italy	HP H7 Ems
1	A/duck/Potsdam/15/1980	I	AJ704797	H7N7 LP	17,03	NEG	NEG	NEG
2	A/duck/Potsdam/13/1980	I	SA	H7N7 LP	17,55	NEG	NEG	NEG
3	A/swan/Potsdam/64/1981	I	AM922155	H7N7 LP	20,07	NEG	NEG	NEG
4	A/turkey/Germany/R11/2001	I	AJ704812	H7N7 LP	18,89	12,74	NEG	NEG
5	A/mallard/NVP/1776–80/2003	I	NAV	H7N3 LP	25,3	16,41	NEG	NEG
6	A/mallard/NVP/41/2004	I	SA	H7N1 LP	15,44	12,49	NEG	NEG
7	A/mallard/Föhr/Wv190/2005	I	NAV	H7N7 LP	27,35	24,10	NEG	NEG
8	A/teal/Föhr/Wv180/2005	I	NAV	H7N2 LP	14,28	10,76	NEG	NEG
9	A/teal/Föhr/Wv177/2005	I	AM933237	H7N7 LP	24,41	21,76	NEG	NEG
10	A/mallard/Germany/R721/2006	I	SA	H7N7 LP	31,38	27,31	NEG	NEG
11	A/graylag goose/Germany/R752/2006	I	AM933236	H7N7 LP	26,15	17,27	NEG	NEG
12	A/mallard/Germany/R756/2006	I	SA	H7N4 LP	24,81	24,13	NEG	NEG
13	A/mute swan/Germany/R57/2006	I	EPI492518	H7N7 LP	27,73	24,20	NEG	NEG
14	A/mute swan/Germany/R901/2006	I	EPI359695	H7N1 LP	23,14	20,08	NEG	NEG
15	A/swan/Germany/736/2006	I	EPI492517	H7N4 LP	15,39	14,07	NEG	NEG
16	A/common pochard/Germany/R916/2006	I	SA	H7N7 LP	19,03	20,32	NEG	NEG
17	A/duck/Germany/R3129/2007	I	SA	H7N7 LP	15,34	11,59	NEG	NEG
18	A/sentinel-duck/Germany/SK207R/2007	I	NAV	H7N3 LP	27,64	22,09	NEG	NEG
19	A/mallard/Sko212-219 K/2007	I	SA	H7N3 LP	25,97	21,04	NEG	NEG
20	A/guineafowl/Germany/R2495/2007	I	AM930528	H7N3 LP	29,58	27,14	NEG	NEG
21	A/mallard/Germany/R192/2009	I	SA	H7N7 LP	14,65	13,28	NEG	NEG
22	A/turkey/Germany/R655/2009	F	EPI302173	H7N7 LP	13,34	11,76	NEG	NEG
23	A/nandu/Germany/AR142/2013	F	SA	H7N7 LP	28,35	28,90	NEG	NEG
24	A/turkey/Germany/AR502/2013	F	SA	H7N7 LP	18,67	19,12	NEG	NEG
25	A/turkey/Germany/AR618/2013	F	NAV	H7Nx LP	16,11	16,20	NEG	NEG
26	A/chicken/Germany/AR909/2013	F	SA	H7Nx LP	35,59	NEG	NEG	NEG
27	A/turkey/Germany/AR979/2013	F	NAV	H7Nx LP	25,59	21,79	NEG	NEG
28	A/environment/Germany/AR1251/2013	F	NAV	H7N LP	21,31	14,93	NEG	NEG
29	A/chicken/Germany/AR929/2015	F, EL	SA	H7N7 LP	30,39	30,02	NEG	NEG
30	A/chicken/Germany/AR930/2015	F, EL	SA	H7N7 LP	30,39	35,77	NEG	NEG
31	A/chicken/Germany/AR934/2015	F, EL	SA	H7N7 LP	30,07	32,88	NEG	NEG
32	A/chicken/Germany/AR943/2015	F, EL	SA	H7N7 LP	30,07	32,70	NEG	NEG
33	A/chicken/Germany/AR944/2015	F, EL	SA	H7N7 LP	30,07	31,03	NEG	NEG
34	A/chicken/Germany/AR945/2015	F, EL	SA	H7N7 LP	29,9	33,18	NEG	NEG
35	A/chicken/Germany/AR946/2015	F, EL	SA	H7N7 LP	29,9	33,32	NEG	NEG
36	A/duck/Germany/AR234/1/2016	F	SA	H7N7 LP	33,42	35,43	NEG	NEG
37	A/duck/Germany/AR2112/2016	F	NAV	H7N7 LP	36,17	*NEG*	NEG	NEG
38	A/duck/Germany/AR2868/2016	F	NAV	H7N7 LP	35,3	*NEG*	NEG	NEG
39	A/FPV/Rostock/45/1934	I	CY077420	H7N1 HP	17,25	NEG	NEG	13,94
40	A/chicken/Germany/"Taucha"/1979	I	SA	H7N7 HP	14,25	NEG	NEG	10,63
41	A/chicken/Germany/R28/2003	I	AJ704813	H7N7 HP	14,77	NEG	NEG	NEG
42	A/FPV/dutch/1927	I	NAV	H7N1 HP	16,52	NEG	NEG	32,14

Table 4 Diagnostic performance characteristics of the H7 pathotyping RT-qPCRs using HP and LP influenza A subtype H7 virus isolates and field samples collected from different countries and poultry holdings or wild bird species, 1999–2016 *(Continued)*

43	A/chicken/Germany/AR1385/2015	F, EL	SA	H7N7 HP	18,76	NEG	NEG	19,01
44	A/chicken/Germany/AR1413/2015	F, EL	SA	H7N7 HP	29,9	NEG	*NEG*	35,48
45	A/chicken/Germany/AR1488/1/2015	F, EL	SA	H7N7 HP	29,31	NEG	NEG	22,72
46	A/environment/Germany/AR1536/2015	F, EL	SA	H7N7 HP	29,38	NEG	NEG	21,18
47	A/environment/Germany/AR1537/2015	F, EL	SA	H7N7 HP	29,38	NEG	NEG	25,7
48	A/environment/Germany/AR1539/2015	F, EL	SA	H7N7 HP	29,38	NEG	NEG	22,19
49	A/environment/Germany/AR1540/2015	F, EL	SA	H7N7 HP	29,38	NEG	NEG	24,69
50	A/environment/Germany/AR1541/2015	F, EL	SA	H7N7 HP	29,38	NEG	*NEG*	26,35
51	A/environment/Germany/AR1546/2015	F, EL	SA	H7N7 HP	30,12	NEG	NEG	25,19
52	A/turkey/Italy/472/1999	I	AJ704811	H7N1 LP	15,24	9,80	NEG	NEG
53	A/chicken/Italy/473/1999	I	EPI624438	H7N2 LP	13,73	10,71	NEG	NEG
54	A/turkey/Italy/2043/2003	I	CY022613, CY022615	H7N3 LP	24,34	21,45	NEG	NEG
55	A/duck/Italy/636/2003	I	NAV	H7N3 LP	22,05	20,49	NEG	NEG
56	A/chicken/Brescia/19/2002	I	AM922154	H7N1 HP	16,59	NEG	*NEG*	NEG
57	A/hen/Italy/444/1999	I	AJ704810	H7N1 HP	16,22	NEG	18,02	NEG
58	A/broiler/Italy/445/1999	I	AJ580353	H7N1 HP	17,02	NEG	16,35	NEG
59	A/turkey/Ireland/PV8/1995	I	AJ704799	H7N7 LP	16,19	13,07	NEG	NEG
60	A/houbara/Dubai/AR433/2014	I	SA	H7N1 LP	16,81	13,51	NEG	NEG
61	A/houbara/Dubai/AR434/2014	I	SA	H7N1 LP	14,67	11,27	NEG	NEG
62	A/houbara/Dubai/AR435/2014	I	SA	H7N1 LP	15,47	12,66	NEG	NEG
63	A/houbara/Dubai/AR436/2014	I	SA	H7N1 LP	12,23	9,23	NEG	NEG
64	A/houbara/Dubai/AR437/2014	I	SA	H7N1 LP	16,1	13,35	NEG	NEG
65	A/houbara/Dubai/AR438/2014	I	SA	H7N1 LP	13,71	10,08	NEG	NEG
66	A/peregrine falcon/Dubai/AR439/2014	I	SA	H7N1 LP	13,79	26,82	NEG	NEG
67	A/francolin/Dubai/AR440/2014	I	SA	H7N2 LP	15,85	17,84	NEG	NEG
68	A/wild bird/Dubai/AR3452/2014	F	SA	H7N1 LP	16,18	14,57	NEG	NEG
69	A/alexandria tyrode/T145/1948	I	SA	H7N1 HP	14,48	NEG	NEG	10
70	A/duck/Alberta/48/1976	I	SA	H7N3 LP	15,8	14,08	NEG	NEG
71	A/turkey/Ontario/18–1/2000	I	AF497552	H7N1 LP	28,61	NEG	NEG	NEG
72	A/mallard/Alberta/8734/2007	I	AM933238	H7N3 LP	18,63	NEG	NEG	NEG
73	A/chicken/BritishColumbia/CN-06/2004	I	KP055066	H7N3 HP	16,42	NEG	NEG	NEG
74	A/chicken/BritishColumbia/CN-07/2004	I	KP055076	H7N3 HP	24,71	NEG	NEG	NEG
75	A/Anhui/1/2013	I	AHZ60096	H7N9 LP	11,79	9,94	NEG	NEG

[a]Sequences were obtained from the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID) and from GenBank at the National Center for Biotechnology Information (NCBI)

LP low pathogenicity, *HP* high pathogenicity, *SA* sequence shown in either Additional file 1: Figure S1 or Additional file 2: Figure S2, otherwise accession numbers are indicated, *NAV* sequence not available, *neg* no positive signal detected, *I* Isolate, *F* Field sample, *F, EL* field sample from recent outbreak in Germany

Conclusion

The pathotype-specific RT-qPCRs developed here for avian influenza viruses of subtype H7 proved to be a useful, sensitive and highly specific alternative to nucleotide sequence analysis for the characterization of LPAI and HPAI H7 viruses of European origin. Proper detection of HP H7 viruses required knowledge of the HACS of the specific lineage, and specific probes are to be used for each distinct lineage. Thus, initial characterization of an H7 HP virus still depends on nucleotide sequence analysis of its HACS. However, in case of on-going spread of the identified HP H7 lineage a lineage-specific probe can then be used in a pathotyping RT-qPCR for the swift examination and pathotyping of further cases and outbreaks. Furthermore, the LP LNA probe introduced here was universally usuable for Eurasian LP H7

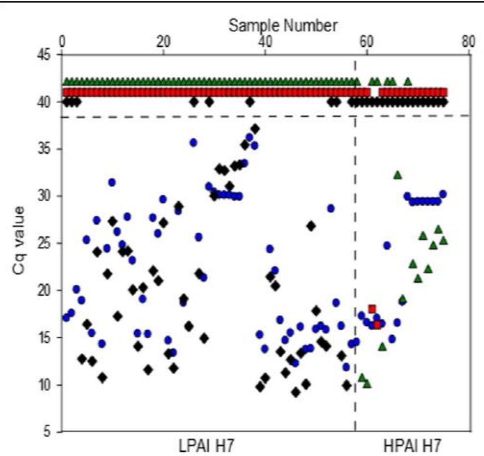


Fig. 4 Sequencing-indpendent pathotyping of isolates and clinical samples of avian influenza subtype H7 viruses by real-time RT-PCRs (RT-qPCR). Sample numbers refer to the identification of viruses in Table 4. Cq values generated for each sample by the influenza A virus-generic M1.2 RT-qPCR are depicted as blue dots. Identification of Cq values obtained for each sample by H7 specific RT-qPCRs is as follows: black diamonds – LPAI H7; green triangles – HPAI H7. 'Emsland'; red squares – HPAI H7 'Italy'

viruses circulating in Europe over the past decade. In conclusion, these here described RT-qPCRs complement a sequencing-independent approach, and allow a high-speed pathotyping helping the authorities to install necessary control measures in time.

Additional file

Additional file 1: Nucleotide sequences encoding the HA endoproteolytic cleavage site of H7N7 low pathogenic avian influenza viruses generated within this study. (PDF 90 kb)

Additional file 2: Nucleotide sequences encoding the HA endoproteolytic cleavage site of H7N7 highly pathogenic avian influenza viruses generated within this study. (PDF 57 kb)

Abbreviations

AIV: Avian influenza virus; CS: Cleavage site; GISAID: Global initiative on sharing Avian influenza data; gs/GD: Goose/Guangdong; HA: Hemagglutinin; HPAIV: Highly pathogenic avian influenza virus; IBV: Infectious bronchitis virus; IVPI: Intravenous pathogenicity index; LPAIV: Low pathogenic avian influenza virus; NA: Neuraminidase; NDV: Newcastle disease virus; RT-qPCR: Reverse transcription real-time PCR

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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

AG and TCH conceived the study. AG carried out the experiments. AG, MB and TCH analysed and interpreted the data. AG drafted the manuscript. All authors amended and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors that they have no competing interests.

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Supplemental material

Additional file 1. Nucleotide sequences encoding the HA endoproteolytic cleavage site of H7N7 low pathogenic avian influenza viruses generated within this study.

Additional file 2. Nucleotide sequences encoding the HA endoproteolytic cleavage site of H7N7 highly pathogenic avian influenza viruses generated within this study.

4.2 PUBLICATION II

Novel real-time PCR-based patho- and phylotyping of potentially zoonotic avian influenza A subtype H5 viruses at risk of incursion into Europe in 2017

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Research article

Novel real-time PCR-based patho- and phylotyping of potentially zoonotic avian influenza A subtype H5 viruses at risk of incursion into Europe in 2017

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Since November 2016, Europe witnesses another wave of incursion of highly pathogenic avian influenza (HPAI) A(H5) viruses of the Asian origin goose/Guangdong (gs/GD) lineage. Infections with H5 viruses of clade 2.3.4.4b affect wild bird and poultry populations. H5 viruses of clades 2.2, 2.3.1.2c and 2.3.4.4a were detected previously in Europe in 2006, 2010 and 2014. Clades 2.2.1.2 and 2.3.2.1.c are endemic in Egypt and Western Africa, respectively and have caused human fatalities. Evidence exists of their co-circulation in the Middle East. Subtype H5 viruses of low pathogenicity (LPAI) are endemic in migratory wild bird populations. They potentially mutate into highly pathogenic phenotypes following transmission into poultry holdings. However, to date only the gs/GD H5 lineage had an impact on human health. Rapid and specific diagnosis marks the cornerstone for control and eradication of HPAI virus incursions. We present the development and validation of five real-time RT-PCR assays (RT-qPCR) that allow sequencing-independent pathotype and clade-specific distinction of major gs/GD HPAI H5 virus clades and of Eurasian LPAI viruses currently circulating. Together with an influenza A virus-generic RT-qPCR, the assays significantly speed up time-to-diagnosis and reduce reaction times in a OneHealth approach of curbing the spread of gs/GD HPAI viruses.

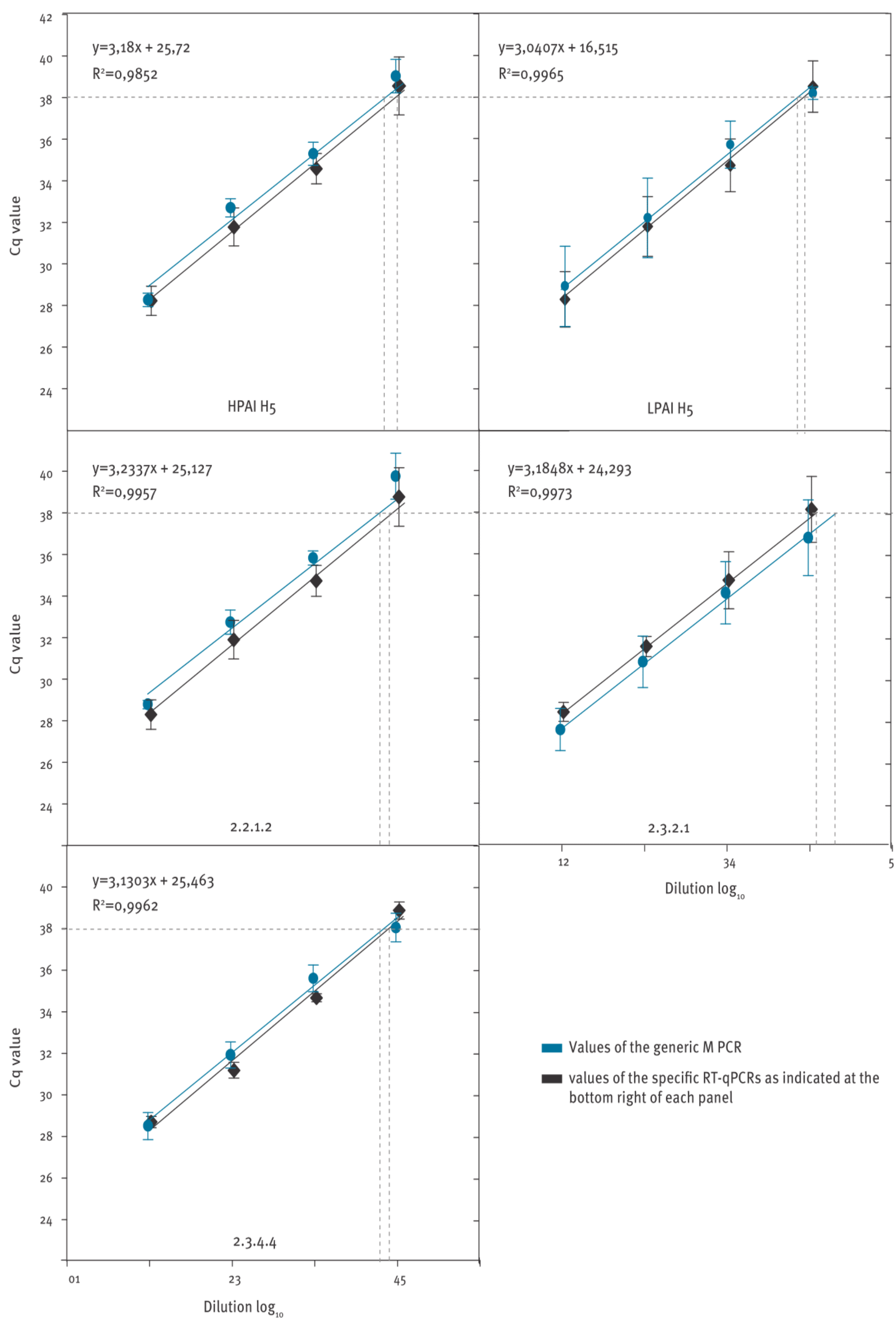
Introduction

Influenza A viruses constitute a virus species in the family *Orthomyxoviridae*. They harbour single-stranded negative-sense RNA arranged into eight genomic segments. Members of this species which infect avian hosts (avian influenza viruses, AIV) are grouped into 16 (H1 to H16) and 9 (N1 to N9) subtypes, respectively, based on phylogenetic and antigenic properties of their haemagglutinin (HA) and neuraminidase (NA) envelope glycoproteins [1]. Different species of aquatic wild birds are the natural reservoirs for all AIV subtypes. Novel subtypes and gene constellations continue to evolve in aquatic wild birds or in infected poultry populations by genetic reassortment during infection of a single host cell with two or more distinct AIV genotypes. In addition to reassortment, the intrinsically error-prone influenza virus genome replication machinery promotes the generation of quasi-species that can be shaped by directional selection pressures, e.g. following host species switches or by specific herd immunity. In the latter case, antigenic drift variants are selected that may escape immunity by very few amino acid substitutions in the HA [2].

Based on their virulence in galliform poultry (e.g. chicken, turkey), AIV are distinguished into groups of highly pathogenic (HP) and low pathogenic (LP) phenotypes [3]. Correct AI diagnosis includes determining the HA subtype and, in case of subtypes H5 or H7, also the pathotype. So far, HPAI phenotypes detected in the field (i.e. 'free' natural environment), were only described among AIV of subtypes H5 and H7 [4]. Some of these viruses including those of the HPAI H5 goose/Guangdong (gs/GD) lineage that emerged in southern China in 1996, have zoonotic potential and are sporadically transmitted from infected birds to humans [5,6]. HPAI viruses of the gs/GD lineage have continued to circulate and evolved into numerous clades. Viruses

Figure 1

Evaluation of detection limits and precision of pathotyping and phylotyping quantitative reverse transcription PCRs compared with a generic matrix (M) gene RT-qPCR[a]



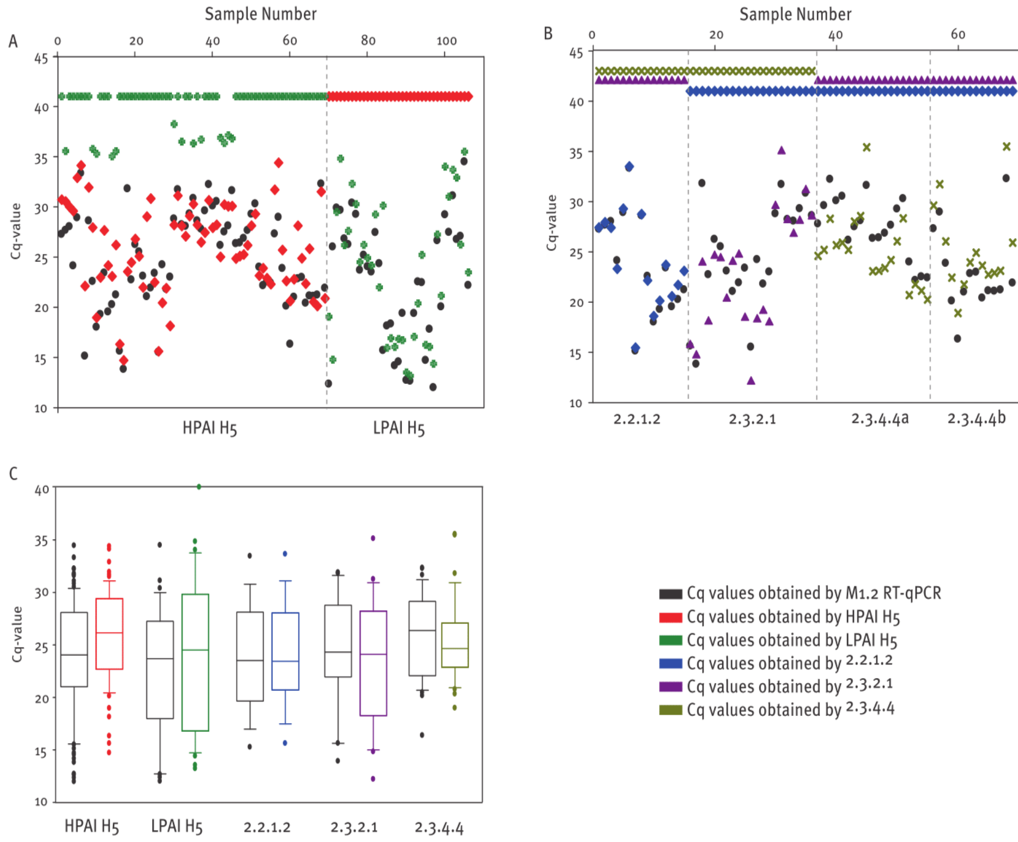
Cq: cycle of quantification; HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza; M: matrix; RT-qPCR: quantitative reverse transcription PCR.

The detection limit was determined based on triplicate analyses of serial 10-fold dilutions of target RNA of reference viruses: HPAI H5: A/duck/Egypt/AR236-A3NLQP/2015 (H5N1); LPAI H5: A/teal-Foehr/Wv1378–79/2003 (H5N2) (upper panel); HPAI H5 clade 2.2.1.2: A/duck/Egypt/AR236-A3NLQP/2015 (H5N1); clade 2.3.2.1: A/quail/Dubai/AR3445–2504.3/2014 (H5N1) and clade 2.3.4.4: A/turkey/Germany-MV/R2472/2014 (H5N8) (lower panel). A cut-off value of Cq 38 was chosen to calculate limits of detection and confidence intervals thereof.

[a] Described in [29].

Figure 2

Pathotyping and phylotyping of virus isolates and clinical samples of potentially zoonotic Eurasian avian influenza A subtype H5 viruses by quantitative reverse transcription PCRs



Cq: cycle of quantification; HPAI: highly pathogenic avian influenza; LPAI: low pathogenic avian influenza; M: matrix; RT-qPCR: quantitative reverse transcription PCR.

Sample numbers in A and B refer to Table 3. Cq values obtained for each sample by M1.2 RT-qPCR are shown as black dots (panels A and B); Cq values obtained for each sample by the specific RT-qPCRs are depicted as follows: Panel A red lozenges – HPAI H5, green crosses - LPAI H5; panel B blue lozenges - clade 2.2.1.2, purple triangles - clade 2.3.2.1, ochre Xs - clade 2.3.4.4.; panel C compares categorised Cq values obtained for all samples by M1.2 RT-qPCR (black box-and-whiskers) and the specific RT-qPCRs (colours as described for panels A and B).

of three major phylogenetic clades (2.2.1.2, 2.3.2.1 and 2.3.4.4) as well as of three further minor clades (1.1.2, 2.1.3.2 and 7.2) have become endemic in poultry populations in several countries in Asia, Africa and the Middle East [7]. Occasionally, spillover transmission from infected poultry may cause infection and viral spread in wild birds with increased mortality in some species. Infected migratory wild birds may spread such viruses across wider distances and act as the source of transmission back to poultry [7,8].

Europe has experienced several incursions by viruses of the gs/GD lineage over the past decade; both wild birds and poultry were affected but no human cases were reported [9]. This is in sharp contrast to Egypt and Asian countries where the endemicity of HPAI H5 viruses in poultry is associated with repeated spillover transmission to and infection of humans. In fact, the majority of human HPAI H5 cases worldwide were registered in Egypt [10,11]. Moreover, a new major clade, designated 2.2.1.2, evolved along with transient spread of an escape mutant-based lineage, 2.2.1.1, in this country [12].

Further potentially zoonotic gs/GD viruses of clade 2.3.2.1c are widespread in Central and Southern Asia and they were sporadically detected along the European Black Sea coast as well as in the Middle East [13-15]. In addition, viruses of this clade have caused major outbreaks among poultry in several Western African countries with ongoing virus circulation to date [16]. Interestingly, 2.3.2.1c viruses have not (yet) been reported from Egypt. Since 2010, another gs/GD cluster, termed 2.3.4.4, has evolved in eastern China

Table 1

Primers and probes designed for differentiating pathotype and phylotype of Eurasian wild bird and goose/Guangdong origin potentially zoonotic avian influenza A subtype H5 viruses

Primer/Probe ID	Target	Sequence (5' to 3')	Location	Amplicon size	Accession number[a]
H5_HP_EA_F1	HPAI H5	CCTTGCDACTGGRCTCAG	984–1001	109	EPI647540
H5_HP_EA_F2		TCCTTGCAACAGGACTAAG	983–1001		
H5_HP_EA_probe		FAM- AAGAARAAARAGAGGACTRTTTGGAGCT-BHQ-1	1023–1050		
H5_HP_EA_R		GTCTACCATTCCYTGCCA	1092–1075		
H5LP-EA_F	LPAI H5	CCCAAATACGTGAAATCAGAT	955–975	133	EPI356413
H5LP1_EA_probe		FAM-CCAAATAGYCCTCTYGTYTCT-BHQ-1	1052–1072		
H5LP-EA_R		GCC ACC CTC CTT CTA TAA AG	1088–1069		
H5_2.2.1.2_Fw	Clade 2.2.1.2	CATTTTGAGAAAATTCAGATCATT	376–399	161	EPI573250
H5_2.2.1.2_probe		FAM-TCCATACCARGGAAGATCCTCCTTT-BHQ-1	451–474		
H5_2.2.1.2_Rev		GGTATGCATCGTTCTTTTTGG	537–517		
H5_2.3.2.1_F	Clade 2.3.2.1	GAGATTGGTACCAAAAATAGCC	669–690	146	EPI603577
H5_2.3.2.1_probe		FAM-ACGGGCAAAGTGGCAGGATAGATTTC-BHQ-1	707–732		
H5_2.3.2.1_R		CAATGAAATTTCCATTACTCTCG	815–793		
H5_2.3.4.4_F_A	Clade 2.3.4.4	ATACCAGGGAGCATCCTCA	484–502	114	EPI554605
H5_2.3.4.4_F_B		ATACCAGGGAACGCCCTCC	484–502		
H5_2.3.4.4_probe		FAM-TCGTTCTTTTTGATGAGCCATACCACA-BHQ-1	540–560		
H5_2.3.4.4_R_A		ATTATTGTAGCTTATCTTTATTGTC	598–574		
H5_2.3.4.4_R_B		ATTATTGTAGCTTATCTTTATTGTT	598–574		

gs/GD: goose/Guangdong; HA: haemagglutinin; ID: identity.

[a] Accession number used to describe the position of the oligonucleotide along the HA gene. Sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI) or the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID).

and on the Korean peninsula [17]. These viruses have revealed a strong propensity to reassort with other influenza subtypes giving rise to novel HPAI sub- and genotypes including influenza A(H5N6) and A(H5N8). The latter subtype has proven to be highly mobile and was carried by infected wild birds to Europe and the North American continent in late 2014 [8,18]. In November 2016, HPAI H5N8 viruses of the 2.3.4.4 clade re-emerged on a large scale in wild birds in several central European countries and caused considerable mortality especially among diving duck species; sporadic incursions into poultry holdings were documented as well [19]. At the same time, this lineage was also detected in poultry in Israel [20].

Eurasian-origin LPAI subtype H5 viruses distantly related to the gs/GD lineage are routinely detected in aquatic wild bird populations with peak incidences during the autumn migration period [21]. Spillover of LPAI virus into poultry may cause notifiable outbreaks and bears the risk of the de novo generation of HP phenotypes following spontaneous mutations [3]. No human LPAI H5 virus infections have been reported so far.

Continuous co-circulation in poultry and sporadic spillover into migratory wild bird populations of different endemic HPAI H5 virus lineages poses constant risks of new incursions into Europe by migrating wild birds or in association with (illegal) poultry trading practices [9]. Furthermore, co-circulation of various HPAI lineages with different antigenic properties potentiates problems of control and eradication. Given the zoonotic propensities of some of the H5 viruses, tight control of infections in poultry is essential to curtail risks of human infections and further spread [22,23]. Molecular diagnosis including patho- and phylotyping of the relevant AIV is an important prerequisite for effective control measures.

We developed rapid diagnostic solutions on the basis of quantitative reverse transcription real-time PCR assays (RT-qPCR), to pathotype, without sequencing, gs/GD lineage HPAI and Eurasian LPAI H5 subtype viruses, and to distinguish HPAI gs/GD viruses of clades 2.2.1.2, 2.3.2.1 and 2.3.4.4, including viruses of the ongoing 2016 epizootic in Europe.

Table 2

Reference viruses used to determine analytical specificity of five PCR assays to detect potentially zoonotic avian influenza subtype H5 viruses

Reference virus		Accession number of HA[a]	Patho- and Phylotype	PCR method [b]				
				HPAI H5	LPAI H5	Clade 2.2.1.2	Clade 2.3.2.1	Clade 2.3.4.4
1	A/turkey/Turkey/1/2005 (H5N1)	KF042153	HP Clade 2.2	Pos	Neg	Pos	Neg	Neg
2	A/chicken/Egypt/0879-NLQP/R737/2008 (H5N1)	GQ184238	HP Clade 2.2.1.1	Pos	Neg	Neg	Neg	Neg
3	A/chicken/Egypt/NLQP7FL-AR747/ 2013 (H5N1)	EPI557170	HP Clade 2.2.1.2	Pos	Neg	Pos	Neg	Neg
4	A/duck/Egypt/AR236-A3NLQP/2015 (H5N1)	EPI573260	HP Clade 2.2.1.2	Pos	Neg	Pos	Neg	Neg
5	A/turkey/Egypt/AR238-SD177NLQP/2014 (H5N1)	EPI573268	HP Clade 2.2.1.2	Pos	Neg	Pos	Neg	Neg
6	A/peregrine falcon/Dubai/AR3430/2014 (H5N1)	EPI603553	HP Clade 2.3.2.1c	Pos	Neg	Neg	Pos	Neg
7	A/quail/Dubai/AR3445–2504.3/2014 (H5N1)	EPI603577	HP Clade 2.3.2.1c	Pos	Neg	Neg	Pos	Neg
8	A/duck/Bangladesh/D3-AR2111/2013 (H5N1)	SA[c]	HP Clade 2.3.2.1a	Pos	Neg	Neg	Pos	Neg
9	A/turkey/Germany/AR2485–86/2014 (H5N8)	EPI552746	HP Clade 2.3.4.4a	Pos	Neg	Neg	Neg	Pos
10	A/turkey/Germany-MV/AR2472/2014 (H5N8)	EPI544756	HP Clade 2.3.4.4a	Pos	Neg	Neg	Neg	Pos
11	A/tufted duck/Germany/AR8444/2016 (H5N8)	EPI859212	HP Clade 2.3.4.4b	Pos	Neg	Neg	Neg	Pos
12	A/chicken/Indonesia/R132/2004 (H5N1)	EPI354072	HP Clade 2.1.1	Pos	Neg	Neg	Neg	Neg
13	A/chicken/Indonesia/R134/2003 (H5N1)	AM183669	HP Clade 2.1.1	Pos	Neg	Neg	Neg	Neg
14	A/chicken/Indonesia/R60/2005 (H5N1)	AM183670	HP Clade 2.1.1	Pos	Neg	Neg	Neg	Neg
15	A/Vietnam/1194/2004 (H5N1)	GQ149236	HP Clade 1.1	Pos	Neg	Neg	Neg	Neg
16	A/chicken/GXLA/1204/2004 (H5N1)	AM183671	HP Clade 2.4	Pos	Neg	Neg	Neg	Neg
17	A/chicken/Vietnam/P41/2005 (H5N1)	AM183672	HP Clade 1.1	Pos	Neg	Neg	Neg	Neg
18	A/chicken/Vietnam/P78/2005 (H5N1)	AM183673	HP Clade 1.1	Pos	Neg	Neg	Neg	Neg
19	A/common teal/Germany/Wv1378–79/2003 (H5N2)	HF563058	LP	Neg	Pos	Neg	Neg	Neg
20	A/duck/Germany/R1789/2008 (H5N3)	CY107849	LP	Neg	Pos	Neg	Neg	Neg
21	A/turkey/Germany/AR915/2015 (H7N7)	SA[c]	H7N7	Neg	Neg	Neg	Neg	Neg
22	A/chicken/Egypt/AR754–14/2013 (H9N2)	EPI557457	H9N2	Neg	Neg	Neg	Neg	Neg
23	A/chicken/Sudan/AR251–15/2014 (IBV)	KX272465	IBV	Neg	Neg	Neg	Neg	Neg
24	A/chicken/Egypt/AR254–15/2014 (NDV)	SA[c]	NDV	Neg	Neg	Neg	Neg	Neg

Cq: cycle of quantification; HA: haemagglutinin; HP: highly pathogenic; HPAI: highly pathogenic avian influenza; IBV: infectious bronchitis virus; LP: low pathogenic; LPAI: low pathogenic avian influenza; NDV: Newcastle disease virus; Neg: negative; Pos: positive; RT-qPCR: quantitative reverse transcription PCR; SA: sequences available.

[a] Sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI) or the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID).

[b] Positive results: Cq value in similar range as with influenza A virus generic M RT-qPCR; negative results: Cq>40. [c] Sequenced in the frame of the current study; sequences available from the authors upon request.

Methods

Virus isolates and clinical samples

A total of 24 reference virus isolates were obtained from the virus repositories at the Friedrich Loeffler Institute, Greifswald-Riems, Germany, or were provided by the National Laboratory for quality control on poultry production in Giza, Egypt, and by the Central Veterinary Research Laboratory (CVRL) in Dubai, United Arab Emirates (see also first table under Results).

Moreover, 106 field samples were included. These were obtained from holdings of different poultry sectors and wild birds from countries in Western Europe (Germany), the Middle East (Egypt, Iraq, United Arab Emirates) and Western Africa (Burkina Faso, Cameroon, Ghana, Ivory Coast, Niger), for HPAI viruses in the period between

Table 3a

Pathotyping and phylotyping of different potentially zoonotic HPAI and LPAI influenza A subtype H5 virus isolates and field samples collected from poultry and wild bird species in different countries, 2013–2016

No.	Sample ID	Type of sample	Accession Number [a]	Clade	PCR results					
					M1.2	HPAI H5	LPAI H5	2.2.1.2	2.3.2.1	2.3.4.4
1	A/chicken/Egypt/NLQP33SD-AR748/2013	Isolate	EPI557178	HP 2.2.1.2	27.25	30.72	Neg	27.43	Neg	Neg
2	A/chicken/Egypt/NLQP2AL-AR749/2013	Isolate	EPI557186	HP 2.2.1.2	27.65	30.53	35.56	27.94	Neg	Neg
3	A/duck/Egypt/NLQP27SG-AR750/2013	Isolate	EPI557194	HP 2.2.1.2	28.01	30.01	Neg	27.41	Neg	Neg
4	A/chicken/Egypt/NLQP639V-AR752/2013	Isolate	EPI557202	HP 2.2.1.2	24.11	29.58	Neg	23.32	Neg	Neg
5	A/chicken/Egypt/NLQP20SL-AR751/2013	Isolate	EPI557210	HP 2.2.1.2	28.90	32.90	Neg	29.30	Neg	Neg
6	A/chicken/Egypt/NLQP139V-AR753/2013	Isolate	EPI557218	HP 2.2.1.2	33.32	34.13	Neg	33.51	Neg	Neg
7	A/quail/Egypt/BSU5514-AR2219/2014	Field sample	EPI557138	HP 2.2.1.2	15.12	22.12	Neg	15.47	Neg	Neg
8	A/chicken/Egypt/AR234-FAOF8NLQP/2014	Field sample	EPI573250	HP 2.2.1.2	28.60	31.95	Neg	28.75	Neg	Neg
9	A/turkey/Egypt/AR235-S240NLQP/2014	Field sample	EPI573252	HP 2.2.1.2	22.56	27.94	35.77	22.16	Neg	Neg
10	A/chicken/Egypt/AR3690A/2016	Field sample	SA[b]	HP 2.2.1.2	18.01	18.97	35.29	18.61	Neg	Neg
11	A/chicken/Egypt/AR3706/2016	Field sample	SA[b]	HP 2.2.1.2	19.27	22.98	Neg	20.13	Neg	Neg
12	A/chicken/Egypt/AR3707/2016	Field sample	SA[b]	HP 2.2.1.2	23.39	27.66	Neg	23.71	Neg	Neg
13	A/chicken/Egypt/AR3737/2016	Field sample	SA[b]	HP 2.2.1.2	19.53	24.16	Neg	20.58	Neg	Neg
14	A/chicken/Egypt/AR3741/2016	Field sample	SA[b]	HP 2.2.1.2	20.25	23.08	35.04	21.71	Neg	Neg
15	A/chicken/Egypt/AR3753/2016	Field sample	SA[b]	HP 2.2.1.2	21.22	26.21	35.55	23.10	Neg	Neg
16	A/seagull/Dubai/AR3443–2504.1/2014	Isolate	EPI603554	HP 2.3.2.1	15.62	16.32	Neg	Neg	15.72	Neg
17	A/stone curlew/Dubai/AR3444–2504.2/2014	Isolate	EPI603569	HP 2.3.2.1	13.81	14.72	Neg	Neg	14.70	Neg
18	A/duck/Ivory_Coast/15VIR2742–1/2015	Spleen and caecum	NA	HP 2.3.2.1	31.79	23.56	Neg	Neg	23.93	Neg
19	A/chicken/Ghana/15VIR2588–4/2015	Spleen	KU97137	HP 2.3.2.1	22.72	24.47	Neg	Neg	18.07	Neg
20	A/chicken/Ghana/15VIR2588–10/2015	Cloacal swab	KU971357	HP 2.3.2.1	26.24	26.80	Neg	Neg	24.61	Neg
21	A/chicken/Niger/15VIR2060–12/2015	Tracheal swab	KU971309	HP 2.3.2.1	25.50	25.08	Neg	Neg	24.37	Neg
22	A/chicken/Niger/15VIR2060–5/2015	Swab	KU971326	HP 2.3.2.1	23.08	21.99	Neg	Neg	20.35	Neg
23	A/domestic_bird/Burkina_Faso/15VIR1774–24/2015	Swab	KU971508	HP 2.3.2.1	21.05	29.03	Neg	Neg	24.01	Neg
24	A/domestic_bird/Burkina_Faso/15VIR1774–23/2015	Organ	KU971500	HP 2.3.2.1	21.91	30.83	Neg	Neg	24.72	Neg
25	A/chicken/Ghana/16VIR-4304–1/2016	Organ	SA[b]	HP 2.3.2.1	23.37	22.49	Neg	Neg	18.44	Neg
26	A/chicken/Ghana/16VIR-4304–25/2016	Organ	SA[b]	HP 2.3.2.1	15.51	15.62	Neg	Neg	12.09	Neg
27	A/chicken/Ghana/16VIR-4304–42/2016	Organ	SA[b]	HP 2.3.2.1	24.22	20.45	Neg	Neg	18.28	Neg
28	A/chicken/Ghana/16VIR-4304–9/2016	Organ	SA[b]	HP 2.3.2.1	21.79	21.90	Neg	Neg	19.13	Neg
29	A/duck/Cameroon/16VIR-3791–21/2016	Lung and trachea	SA[b]	HP 2.3.2.1	23.00	18.14	Neg	Neg	17.98	Neg
30	A/chicken/Iraq/AR5282/2016	Field sample	NA	HP 2.3.2.1	28.78	28.20	Neg	Neg	29.57	Neg
31	A/chicken/ Iraq/AR5283/2016	Field sample	NA	HP 2.3.2.1	31.70	31.12	Neg	Neg	35.02	Neg
32	A/chicken/Iraq/AR5286/2016	Field sample	SA[b]	HP 2.3.2.1	28.21	28.10	36.50	Neg	28.16	Neg
33	A/chicken/Iraq/AR5287/2016	Field sample	SA[b]	HP 2.3.2.1	28.05	27.08	Neg	Neg	26.80	Neg
34	A/chicken/Iraq/AR5291/2016	Field sample	SA[b]	HP 2.3.2.1	29.29	29.09	Neg	Neg	28.09	Neg
35	A/chicken/Iraq/AR5292/2016	Field sample	NA	HP 2.3.2.1	30.83	30.28	36.32	Neg	31.15	Neg
36	A/chicken/Iraq/AR5296/2016	Field sample	SA[b]	HP 2.3.2.1	28.60	28.21	Neg	Neg	28.53	Neg
37	A/turkey/Germany/AR2499/2014	Field sample	SA[b]	HP 2.3.4.4	27.78	26.48	36.71	Neg	Neg	24.61
38	A/turkey/Germany/AR2500/2014	Field sample	SA[b]	HP 2.3.4.4	29.59	27.44	Neg	Neg	Neg	25.20
39	A/turkey/Germany/AR2501/2014	Field sample	SA[b]	HP 2.3.4.4	32.21	30.65	Neg	Neg	Neg	28.30
40	A/turkey/Germany/AR2502/2014	Field sample	SA[b]	HP 2.3.4.4	30.08	27.92	Neg	Neg	Neg	25.67
41	A/turkey/Germany/AR2503/2014	Field sample	SA[b]	HP 2.3.4.4	30.52	28.21	Neg	Neg	Neg	25.92
42	A/turkey/Germany/AR2562/2014	Field sample	SA[b]	HP 2.3.4.4	26.15	25.02	36.88	Neg	Neg	25.21
43	A/turkey/Germany/AR2574/2014	Field sample	SA[b]	HP 2.3.4.4	27.49	30.23	36.36	Neg	Neg	28.01
44	A/turkey/Germany/AR2591/2014	Field sample	SA[b]	HP 2.3.4.4	28.09	30.06	37.13	Neg	Neg	28.57
45	A/teal/Germany/AR2917/2014	Field sample	SA[b]	HP 2.3.4.4	31.60	30.08	36.82	Neg	Neg	35.41
46	A/turkey/Germany/AR3372/2014	Field sample	EPI553172	HP 2.3.4.4	26.33	24.85	Neg	Neg	Neg	23.07
47	A/turkey/Germany/AR3376/2014	Field sample	SA[b]	HP 2.3.4.4	26.39	25.10	Neg	Neg	Neg	23.12
48	A/turkey/Germany/AR3381/2014	Field sample	SA[b]	HP 2.3.4.4	26.85	25.26	Neg	Neg	Neg	23.40
49	A/turkey/Germany/AR3382/2014	Field sample	SA[b]	HP 2.3.4.4	27.64	26.18	Neg	Neg	Neg	24.18
50	A/turkey/Germany/AR3383/2014	Field sample	SA[b]	HP 2.3.4.4	29.26	28.13	Neg	Neg	Neg	26.06
51	A/duck/Germany/AR3457/2014	Field sample	SA[b]	HP 2.3.4.4	30.29	29.30	Neg	Neg	Neg	28.34
52	A/duck/Germany/AR3465/2014	Field sample	SA[b]	HP 2.3.4.4	23.98	23.15	Neg	Neg	Neg	20.70
53	A/duck/Germany/AR3470/2014	Field sample	SA[b]	HP 2.3.4.4	22.15	23.89	Neg	Neg	Neg	21.78

HA: haemagglutinin; HP: highly pathogenic; HPAI: highly pathogenic avian influenza; ID: identity; LP: low pathogenic; LPAI: low pathogenic avian influenza; NA: sequence not available; Neg: negative; SA: sequence available.

[a] Sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI) or the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID).

[b] Sequenced in the frame of the current study; sequences available from the authors upon request.

2013 and 2016. Samples consisted mainly of oropharyngeal and/or cloacal swabs and tissues samples (n = 70) or AIV isolated from such samples (n = 36) (see also second table under Results).

A subsection of the 106 clinical samples (n = 13) was provided as dried material on Whatman FTA card (Sigma Aldrich, Germany). Samples from Western African countries were exclusively assayed at the Istituto Zooprofilattico Sperimentale delle Venezie, Padua, Italy.

Design of primers and probes

Primers were chosen based on alignments of the HA H5 gene of a selection of influenza A virus sequences submitted over the past 10 years to GenBank at the National Center for Biotechnology Information (NCBI) or to the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID). Selected sequences represented Eurasian LP viruses and HP isolates and clades of the gs/GD lineage that were detected in Europe, the Middle East and Western Africa during the past decade. Selection of primers to amplify a small fragment of the HA gene spanning the endoproteolytic cleavage site aimed at being broadly inclusive so as to target as many of the published LP Eurasian H5 HA sequences as possible and to distinguish them from HP viruses of the gs/GD lineage. The probes were placed directly onto the cleavage site in the attempt to specifically bind to sequences encoding either mono- or polybasic patterns that distinguish LP and HP pathotypes, respectively (Table 1).

At first, sets of primers and probes were designed to detect and discriminate between HP and LP biotypes, i.e. Eurasian H5 viruses encoding a monobasic or a polybasic HA cleavage site. In addition, four different sets of primers and probes were developed to differentiate between gs/GD clades 2.2.1.2, 2.3.2.1 and 2.3.4.4 (A and B). Pre-selected primers were then screened in silico for their specificity properties using Shannon entropy plots implemented in the Entropy One software (http://www.hiv.lanl.gov/content/sequence/-ENTROPY/entropy_one.html). Oligont (oligont) were selected so as to retain full specificity for the selected clade and to maximise entropy against all other clades. Basic physical properties of oligont were checked using the online web interface Oligo Calculator version 3.27. The finally chosen oligont are shown in Table 1. Detailed results of the in silico analyses are available on request from the authors.

One-step quantitative reverse transcription PCR assays

All reactions were performed using the AgPath-ID One-Step RT-qPCR kit (Thermofisher, scientific, United States) as follows: Reverse transcription at 45 °C for 10 min, initial denaturation at 95 °C for 10 min, 40 cycles of PCR amplification at 95 °C for 30 s, 58 °C for 15 s, and 72 °C for 15 s in a 25 µl reaction mixture using 15 pmol of each forward and reverse primers and 5 pmol probe per reaction. For each parameter a separate reaction was used. Cycling was performed on a Biorad CFX96 Real-Time cycler (BioRad, Germany). Fluorescent signals were collected during the annealing phase, and the amplification data were analysed using Bio-Rad CFX Manager 3 software accessing automated fluorescence drift correction for baseline adjustment.

Nucleotide sequencing and clade assignment

Patho- and phylotyping results obtained by newly developed RT-qPCRs were counter-checked by nt (nt) sequencing of the entire or parts of the HA gene of the respective isolates/clinical samples. Amplification of the HA gene was performed using primers published previously [24] and primers recommended in the European Union Diagnostic Manual for AI in a one-step RT-PCR [25]. In addition, amplificates of the HPAI H5 and LPAI H5 RT-qPCRs were used for sequencing purposes as well. Products were size-separated in agarose gels, excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Purified PCR products were used for cycle sequencing reactions (BigDye Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems, California, United States) the products of which were purified using NucleoSEQ columns (Macherey-Nagel GmbH and Co, Düren, Germany) and sequenced on an ABI PRISM 3130 Genetic Analyzer (Life Technologies, Darmstadt, Germany).

For pathotyping, deduced amino acid sequences of the endoproteolytical cleavage site of the HA gene were inspected and compared with the molecular pathotyping database provided by OFFLU [26]. Assignment of nt sequences to the gs/GD HPAI H5 virus clade system was performed by use of clade prediction tool implemented in the Influenza Research Database [27].

Results

Analytical specificity of pathotyping and phylotyping quantitative reverse transcription PCR assays

The specificity of the assays was evaluated with viral RNA from representative influenza A subtype H5 viruses that had been phylotyped based on full-length HA nt sequence analysis (Table 2). Furthermore, non-H5 subtypes, i.e. H9N2 and H7N7, as well as non-influenza avian viruses i.e. avian infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) were employed (Table 2), and none of them was detected by any of the specific PCRs.

In the initial evaluation of the specificity of the pathotyping RT-qPCR assays carried out using two reference viruses: HPAI A/chicken/Egypt/AR236/2015 (H5N1, clade 2.2.1.2) and LPAI A/turkey/Germany/R2025/2008 (H5N3), specific reactivity exclusively with the homopathotypic virus was evident. In a second step, assays were extended to the full range of 24 reference viruses yielding a similar sharp distinction between HP and LP cleavage sites (Table 2).

Table 3B

Pathotyping and phylotyping of different potentially zoonotic HPAI and LPAI influenza A subtype H5 virus isolates and field samples collected from poultry and wild bird species in different countries, 2013–2016

No.	Sample ID	Type of sample	Accession Number[a]	Clade	PCR results					
					M1.2	HPAI H5	LPAI H5	2.2.1.2	2.3.2.1	2.3.4.4
54	A/wild-duck/Germany/AR8603/2016	Field sample	SA[b]	HP 2.3.4.4b	22.51	22.90	Neg	Neg	Neg	21.14
55	A/greyleg goose /Germany/AR8604/2016	Field sample	SA[b]	HP 2.3.4.4b	22.41	22.30	Neg	Neg	Neg	20.26
56	A/greater scaup/Germany/AR9090/2016	Field sample	SA[b]	HP 2.3.4.4b	27.29	31.71	Neg	Neg	Neg	29.61
57	A/greater scaup/Germany/AR9091/2016	Field sample	SA[b]	HP 2.3.4.4b	28.95	34.40	Neg	Neg	Neg	31.74
58	A/greater scaup/Germany/AR9092/2016	Field sample	SA[b]	HP 2.3.4.4b	23.85	25.70	Neg	Neg	Neg	26.05
59	A/grey heron/Germany/AR9093/2016	Field sample	SA[b]	HP 2.3.4.4b	20.10	22.62	Neg	Neg	Neg	22.44
60	A/greater scaup/Germany/AR9094/2016	Field sample	SA[b]	HP 2.3.4.4b	16.31	20.62	Neg	Neg	Neg	18.92
61	A/greater scaup/Germany/AR9095/2016	Field sample	SA[b]	HP 2.3.4.4b	20.99	22.79	Neg	Neg	Neg	21.75
62	A/northern pintail /Germany/AR9096/2016	Field sample	SA[b]	HP 2.3.4.4b	22.83	28.12	Neg	Neg	Neg	23.95
63	A/bean goose/Germany/AR9097/2016	Field sample	SA[b]	HP 2.3.4.4b	22.97	24.88	Neg	Neg	Neg	24.92
64	A/herring gull /Germany/AR9098/2016	Field sample	SA[b]	HP 2.3.4.4b	20.40	22.35	Neg	Neg	Neg	23.64
65	A/mute swan/Germany/AR9099/2016	Field sample	SA[b]	HP 2.3.4.4b	21.12	25.83	Neg	Neg	Neg	22.75
66	A/chicken/Germany/AR9140/2016	Field sample	SA[b]	HP 2.3.4.4b	21.08	20.55	Neg	Neg	Neg	22.92
67	A/chicken/Germany/AR9141/2016	Field sample	SA[b]	HP 2.3.4.4b	21.21	20.13	Neg	Neg	Neg	23.12
68	A/chicken/Germany/AR9143/2016	Field sample	SA[b]	HP 2.3.4.4b	32.27	31.51	Neg	Neg	Neg	35.49
69	A/chicken/Germany/AR9144/2016	Field sample	SA[b]	HP 2.3.4.4b	21.89	20.89	Neg	Neg	Neg	25.92
70	A/chicken/Italy/22/1998	Isolate	CAP58165	LPAI H5N9	12.34	Neg	19.06	Neg	Neg	Neg
71	A/mallard/Germany/Wv1349–51K/2003	Isolate	CAP58164	LPAI H5N3	26.00	Neg	14.79	Neg	Neg	Neg
72	A/mallard/Germany/Wv476/2004	Isolate	NA	LPAI H5N2	29.87	Neg	29.5	Neg	Neg	Neg
73	A/mallard/Germany/Wv474–77K/2004	Isolate	NA	LPAI H5N2	29.64	Neg	34.81	Neg	Neg	Neg
74	A/ostrich/Germany/R5–10/2006	Isolate	HF563057	LPAI H5N3	26.80	Neg	26.19	Neg	Neg	Neg
75	A/mallard/Germany/R2557/2006	Isolate	NA	LPAI H5N3	26.24	Neg	27.61	Neg	Neg	Neg
76	A/mallard/Germany/R731/2008	Isolate	SA[b]	LPAI H5N3	30.36	Neg	32.30	Neg	Neg	Neg
77	A/mallard/Germany/R771/2008	Isolate	SA[b]	LPAI H5N3	29.24	Neg	30.28	Neg	Neg	Neg
78	A/mallard/Germany/R772/2008	Isolate	SA[b]	LPAI H5N3	23.68	Neg	24.50	Neg	Neg	Neg
79	A/turkey/Germany/R1550/2008	Isolate	NA	LPAI H5N3	25.17	Neg	26.22	Neg	Neg	Neg
80	A/turkey/Germany/R1551/2008	Isolate	NA	LPAI H5N3	24.03	Neg	24.91	Neg	Neg	Neg
81	A/turkey/Germany/R1557/2008	Isolate	SA[b a]	LPAI H5N3	23.50	Neg	24.16	Neg	Neg	Neg
82	A/turkey/Germany/R1612/2008	Isolate	NA	LPAI H5N3	27.43	Neg	29.25	Neg	Neg	Neg
83	A/turkey/Germany/R2014/2008	Isolate	SA[b]	LPAI H5N3	24.33	Neg	21.99	Neg	Neg	Neg
84	A/turkey/Germany/R2015/2008	Isolate	SA[b]	LPAI H5N3	15.69	Neg	30.13	Neg	Neg	Neg
85	A/turkey/Germany/R2016/2008	Isolate	SA[b]	LPAI H5N3	18.13	Neg	15.96	Neg	Neg	Neg
86	A/turkey/Germany/R2017/2008	Isolate	SA[b]	LPAI H5N3	18.32	Neg	16.92	Neg	Neg	Neg
87	A/turkey/Germany/R2018/2008	Isolate	SA[b]	LPAI H5N3	14.16	Neg	16.05	Neg	Neg	Neg
88	A/turkey/Germany/R2019/2008	Isolate	SA[b]	LPAI H5N3	14.55	Neg	16.84	Neg	Neg	Neg
89	A/turkey/Germany/R2020/2008	Isolate	SA[b]	LPAI H5N3	19.38	Neg	16.73	Neg	Neg	Neg
90	A/turkey/Germany/R2021/2008	Isolate	SA[b]	LPAI H5N3	12.71	Neg	13.51	Neg	Neg	Neg
91	A/turkey/Germany/R2022/2008	Isolate	SA[b]	LPAI H5N3	12.63	Neg	13.18	Neg	Neg	Neg
92	A/turkey/Germany/R2023/2008	Isolate	SA[b]	LPAI H5N3	19.37	Neg	17.07	Neg	Neg	Neg
93	A/turkey/Germany/R2024/2008	Isolate	SA[b]	LPAI H5N3	22.52	Neg	20.39	Neg	Neg	Neg
94	A/turkey/Germany/R2025/2008	Isolate	SA[b]	LPAI H5N3	22.44	Neg	25.22	Neg	Neg	Neg
95	A/turkey/Germany/R2026/2008	Isolate	SA[b]	LPAI H5N3	14.70	Neg	16.26	Neg	Neg	Neg
96	A/turkey/Germany/R2027/2008	Isolate	SA[b]	LPAI H5N3	17.80	Neg	16.06	Neg	Neg	Neg
97	A/mallard/Germany/R2892–94/2009	Isolate	EPI356412	LPAI H5N3	11.98	Neg	14.37	Neg	Neg	Neg
98	A/duck/Germany/AR1965/2013	Field sample	NA	LPAI H5N3	26.62	Neg	27.25	Neg	Neg	Neg
99	A/turkey/Germany/AR1892/1/2014	Field sample	SA[b]	LPAI H5N2	20.03	Neg	21.15	Neg	Neg	Neg
100	A/duck/Germany/AR1/2015	Field sample	SA[b]	LPAI H5N3	29.20	Neg	34.01	Neg	Neg	Neg
101	A/swan/Germany/AR111/2015	Field sample	SA[b]	LPAI H5N4	27.45	Neg	31.02	Neg	Neg	Neg
102	A/goose/Germany/AR398/2015	Field sample	SA[b]	LPAI	31.09	Neg	33.69	Neg	Neg	Neg
103	A/duck/Germany/AR1231/1/2015	Field sample	NA	LPAI H5N2	26.74	Neg	32.91	Neg	Neg	Neg
104	A/duck/Germany/AR2853/15–1/2015	Field sample	SA[b]	LPAI H5N3	27.06	Neg	26.25	Neg	Neg	Neg
105	A/goose/Germany/AR3264/1/2015	Field sample	SA[b]	LPAI H5N2	34.47	Neg	35.50	Neg	Neg	Neg
106	A/wild bird/Germany/AR221/2015	Field sample	SA[b]	LP H5N3	22.17	Neg	23.48	Neg	Neg	Neg

HA: haemagglutinin; HP: highly pathogenic; HPAI: highly pathogenic avian influenza; ID: identity; LP: low pathogenic; LPAI: low pathogenic avian influenza; NA: sequence not available; Neg: negative; SA: sequence available.

[a] Sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI) or the EpiFlu database of the Global Initiative on Sharing Avian Influenza Data (GISAID).

[b] Sequenced in the frame of the current study; sequences available from the authors upon request.

Primers and probes for phylotyping RT-qPCR assays distinguishing three clades of gs/GD origin HPAIV H5 were placed within the HA1-fragment of the HA gene. This region encodes the receptor binding unit and harbours a number of neutralisation-relevant epitopes that are targets of antigenic drift. Hence, the HA1 fragment harbours regions that are the least conserved within the influenza A virus genome. Primer selection aimed at the inclusion of as many as possible distinguishing nt that would define exclusivity at the five most 3' positions while probes were placed so as to accommodate distinguishing nt in the centre of the oligont. In order not to compromise amplification efficacy, amplicon size was limited to 130 nt wherever possible given the above mentioned constraints for primers and probes. The finally chosen oligont are listed in Table 1 and provided specific detection exclusively of the homologous clade. No cross-reactivity among the other gs/GD clades examined was evident on basis of the used panel of reference viruses (Table 2). Also, no cross-reactivity was detected for any of the five assays against other influenza A viruses or other avian viral respiratory pathogens (Table 2).

Validation of the analytical sensitivity, limit of detection and precision

Detection limits of the assays were determined by testing 10-fold serial dilutions of viral RNA extracted from representative viruses of each of the three HPAI virus clades (2.2.1.2, 2.3.2.1 and 2.3.4.4), and of Eurasian H5 LPAI virus. Cycle of quantification (Cq) values were compared with a standard RT-qPCR for the matrix (M) gene of these viruses with a reported detection limit of 2 to 20 RNA copies/5 µl [28]. Average values of three separate runs were computed and plotted using SigmaPlot V 11 software. Plotting these values revealed a linear relationship between the log of the viral RNA dilution and the Cq value for all assays and the kinetics of the assays and their sensitivity were determined to be very similar to the generic M gene RT-qPCR (M1.2 RT-qPCR [29]) (Figure 1).

The correlation coefficient of the standard curves was 0.99 for all assays, indicating a highly precise log–linear relationship between the viral RNA log dilution and the corresponding Cq-value (Figure 1). Based on these results the threshold distinguishing positive and negative was set at Cq = 38.

Pathotyping and phylotyping of clinical samples of potentially zoonotic Eurasian avian influenza A subtype H5 viruses by quantitative reverse transcription PCR

In order to evaluate the diagnostic performance capacity of the developed assays, field samples (RNA extracted from swabs, tissues or FTA cards) and clinical virus isolates obtained during the period 2013 to 2016 (HPAI viruses) or 2003 to 2015 (LPAI viruses) were examined. The sample set was preselected on basis of a positive generic M-specific RT-qPCR.

Among the final set of 106 samples, the pathotyping RT-qPCRs sharply discerned two groups of 69 samples reacting only in the new HPAI H5 RT-qPCR while 37 samples reacted positive in the LPAI H5 RT-qPCR (Figure 2a; Table 3).

All pathotyping results matched the results obtained by nt sequence analysis of the HA cleavage site. However, in a few samples (two isolates, 10 clinical samples) of HP viruses, the LPAI H5 RT-qPCR also gave a weak positive signal (Cq > 35). Compared with the LPAI H5 signal the HPAI H5 signal of these samples yielded Cq values 6–10 units lower on average ascertaining good diagnostic specificity. Depending on the clade, the HP phenotype was detected with equal (clade 2.3.2.1) or slightly reduced (clade 2.2.1.2) sensitivity; the LP H5 RT-qPCR appeared to be slightly less sensitive than the M PCR as far as clinical samples were concerned (Table 3; Figure 2a and c). Sequences across the cleavage sites of these samples are presented in a supplemental alignment (Figure 2).

In a next step, the samples that were designated HPAI H5-positive were subjected to the three phylotyping RT-qPCRs. Here, 15, 21 and 33 samples, respectively, were exclusively positive for either clade 2.2.1.2, 2.3.2.1 or 2.3.4.4 (Table 3). Thus, a clear cut clade assignment was possible for all gs/GD HP H5 samples. Results were counterchecked by feeding available HA sequences of these samples into the IRD clade prediction tool (www.fludb.org/brc/h5n1-Classifier.spg?method=ShowCleanInputPage&decorator=influenz): In all cases the same clade was assigned by sequence analysis and by PCR. In a final step also all LPAI H5 samples were tested in the phylotyping RT-qPCRs and none of them cross-reacted. Regarding the sensitivity of these PCRs, the Cq values were compared with those of the generic M1.2-specific RT-qPCR (Figure 2b). For clade 2.2.1.2 and 2.3.2.1 the sensitivity was almost identical to the M PCR; for clade 2.3.4.4a, the clade-specific PCR proved to be slightly more sensitive while viruses of clade 2.3.4.4b were detected at a slightly lower sensitivity; detection of clade 2.3.4.4b viruses was slightly less sensitive than the M PCR (Figure 2b and c; Table 3) as far as clinical samples were concerned.

Rank Sum tests implemented in the SigmaPlot software package were performed and no statistically significant difference between the median Cq values of each specific assay and the M1.2 RT-qPCR assay was found (p > 0,50) indicating that the newly developed RT-qPCRs display similar analytical sensitivity. Thus, the phylotyping RT-qPCRs allow a sensitive and highly specific detection and distinction of the three major gs/GD clades currently circulating in countries where the viruses were obtained from.

Discussion

Rapid molecular diagnosis including patho- and phylotyping is basis to enable measures aimed at repressing

the spread of potentially zoonotic HPAI viruses. The TaqMan PCR technology has proven reliable, versatile, and comparatively cost-effective in the generic detection and subtype differentiation of AIV [30]. Further differentiation of clades, lineages and pathotypes was previously nearly entirely based on nt sequencing approaches which require expensive equipment and are time consuming. In epidemiologically complex settings where different lineages and pathotypes of potentially zoonotic and notifiable infectious agents co-circulate, a more rapid and direct access to testing and results, e.g. by using RT-qPCRs, is desirable. Although RT-qPCRs are inferior to sequencing techniques in terms of retrievable data details, they are superior with respect to time-to-diagnosis and ease-of-use. This concept which we used earlier for pathotyping of H5N1 [31], was here further extended and refined for the identification and discrimination of avian influenza A subtype H5 viruses of different patho- and phylotypes. The focus was put on those clades of H5 viruses (2.2.1.2, 2.3.2.1, 2.3.4.4) that had previously 'escaped' from Asia and were detected in western parts of Eurasia and in Africa.

Pathotyping of avian influenza A subtype H5 viruses is mandatory from an animal health perspective. The pathotyping RT-qPCRs presented here reduce time-to-diagnosis to just three hours following sample receipt. To our knowledge this is the broadest and most detailed attempt of AIV pathotyping using RT-qPCR. The availability of highly sensitive pathotyping PCRs would also allow to detect mixtures of HP and LP H5 viruses in the same sample; in fact, some of our HP-positive field samples also gave weak LP signals (Table 3, sample numbers 2, 9, 10, 14, 15, 30, 32, 35, 37, 42–5). Yet, LPAI pathotypes in these samples were detected at distinctly higher Cq values indicating either a minor population in a quasispecies of different pathotypes or expressing some cross-reactivity of LPAI primers and probe; in any case, the detection of HPAI genotypes as a major population in a set of field samples was always unequivocal. Further insight into the true nature of these mixtures would only be unravelled by deep sequencing approaches of those samples.

Rapid pathotyping enables rapid implementation of appropriate measures to prevent further spread of virus such as closure of poultry holdings and/or live poultry markets, culling of infected flocks etc. This impedes accumulation of potentially zoonotic AIV at the poultry-human interface which in turn lowers the risks of human infection.

Phylotyping of gs/GD HPAI H5 virus clades is important since each clade, and often also sublineages thereof, display distinct antigenic and pathogenetic properties. This has direct implications, as by assigning the matching clade, appropriate vaccines that ensure the closest antigenic match with the circulating viruses can be selected [32,33]. In particular, countries where gs/GD viruses have become endemic in poultry populations, rely on vaccination of poultry on a broad scale to suppress circulating viruses and to limit risks of human exposure [23]. However, it should be noted that mutant escape variants within these clades selected by vaccine-induced population immunity will not be detected as such by the assays, and in fact, such mutants may also be detected at lower sensitivity if primer and/or probe binding sites are affected by mutations. Detection of variants will still depend on either nt sequencing or virus isolation/antigenic characterisation approaches but the newly developed assays will aid in selection of meaningful samples in this respect. In particular, samples that do not give conclusively similar Cq values in the generic and the specific assays should prompt in-depth analysis by nt sequencing.

It should be clearly stated that the assays presented here have limitations owed to the restricted geographical distribution of the targeted clades. The use of the newly developed PCRs in regions where viruses belonging to the targeted clades (2.2.1.2, 2.3.2.1c and 2.3.4.4) are reportedly absent is only recommended if immediate incursions with any of these clades are apprehended. Phylotyping indirectly may point towards zoonotic potential since different gs/GD lineages vary in their zoonotic propensity: Egyptian 2.2.1.2 viruses are characterised by increased affinity to human-like sialic acid receptors and have caused by far the largest number of human influenza A(H5N1) virus infections over the past decade [12]. For clade 2.3.2.1c viruses, repeatedly detected in the Middle East (excluding Egypt) and endemic in Western African countries, only few human cases have been recorded. The 2.3.4.4 viruses currently present in various parts of Europe have not provoked human infection so far [34].

Extended co-circulation of more than one gs/GD lineage in poultry and/or wild birds in a wider geographic region was repeatedly reported [35,36]. It is pivotal, for the above mentioned reasons, to detect incursions of distinct HPAI virus lineages in a timely manner. In this respect, the newly developed RT-qPCR assays were shown to be useful tools for an improved rapid and simple characterisation of patho- and phylotypes of Eurasian origin avian influenza A subtype H5 viruses. The assays aid in speeding up diagnosis on clinical samples because the time consuming (initial) need of virus isolation and nt sequencing is avoided. Given the high substitution rate of HP H5 influenza viruses frequent checks and, if required, updates of the primers and probes are recommended to ensure full specificity and sensitivity of the patho- and phylotyping RT-qPCRs. These PCRs are advantageous in particular for wild bird samples, especially those that contain LPAI viruses, often with low viral loads and therefore fail to yield replication-competent virus. With respect to HPAI virus, the renouncement from initial virus isolation improves biosecurity. However, the presented assays are not intended to replace virus isolation and antigenic characterisation as a means to detect emerging antigenic drift mutants. Nevertheless, they may aid in selection

of appropriate samples for such tasks. Accurate phylotyping also facilitates selection of appropriate vaccines as it serves as an early warning for the incursion of new and antigenically possibly distinct phylotypes.

Conclusions

The assays reported here are primarily intended for screening purposes of avian samples; confirmatory assays, including nt sequence analyses and antigenic characterisation, are still required for new incursions and outbreak scenarios that feature an expansion of the geographic area and/or the range of affected species or poultry sectors. When used in the frame of on-going outbreaks, in particular in regions where vaccination is not used as a preventive measure, results of the patho- and phylotyping PCRs are deemed solid enough for reporting purposes and to justify the implementation of restriction measures. In such settings, similar to the current outbreaks of clade 2.3.4.4b HP H5N8 in Europe, the assays can be prioritised to running the HP and only one (i.e. the fitting) of the phylotyping PCRs on M1.2- and H5 PCR-positive samples. This significantly speeds up time-to-diagnosis and reduces reaction times in a OneHealth approach of repressing the spread of gs/GD HP AIV. Sequencing facilities, classically required for patho- and phylotyping, may not be available, and even not logistically accessible in many regions severely affected by H5 HPAI incursions. The prospect of having sequencing-independent, TaqMan-based specific and sensitive typing assays, as described here, available in developing regions is expected to boost regional diagnostic capacities eventually leading to improved disease control.

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Conflict of interest

None declared. One of the authors (N.A.) works for Boehringer Ingelheim, Dubai, United Arab Emirates.

Authors' contributions

Mahmoud M. Naguib, Annika Graaf and Timm Harder conceived the study. Andrea Fortin, Ulrich Wernery, Nadim Amarin, Hussein A. Hussein, Hesham Sultan and Basem Al Adhadh were involved in the collection, initial analysis and provision of viruses and field samples. Christine Luttermann conducted the Sanger sequencing analyses. Mahmoud M. Naguib, Annika Graaf and Andrea Fortin produced, analysed and interpreted data. Timm Harder and Mahmoud M. Naguib drafted the manuscript. Isabella Monne, Martin Beer and all co-authors critically analysed and revised the manuscript and provided final approval.

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4.3 PUBLICATION III

From low to high pathogenicity—Characterization of H7N7 avian influenza viruses in two epidemiologically linked outbreaks

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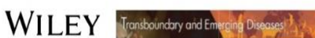

From low to high pathogenicity—Characterization of H7N7 avian influenza viruses in two epidemiologically linked outbreaks

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Abstract

The ability of low pathogenic (LP) avian influenza viruses (AIV) of the subtypes H5 and H7 to mutate spontaneously to highly pathogenic (HP) variants is the main reason for their stringent control. On-the-spot evidence from the field of mutations in LPAIV to render the virus into nascent HP variants is scarce. Epidemiological investigations and molecular characterization of two spatiotemporally linked outbreaks caused by LP, and subsequently, HPAIV H7N7 in two-layer farms in Germany yielded such evidence. The outbreaks occurred within 45 days on farms 400 m apart. The LP progenitor virus was identified on both farms, with its putative HP inheritor cocirculating and then dominating on the second farm. As postulated before, mutations in the hemagglutinin cleavage site (HACS) proved to be the most decisive change in the genome of HPAIV, in this case, it was mutated from monobasic (LP) PEIPKGR*GLF into polybasic (HP) PEIPKRKRR*GLF. The full-length genome sequences of both viruses were nearly identical with only ten coding mutations outside the HACS scattered along six genome segments in the HPAIV. Five of these were already present as minor variants in the LPAIV quasispecies of the LPAI-only affected farm. H7-specific seroconversion of part of the chicken population together with the codetection of LPAIV HACS sequences in swab samples of the HPAI outbreak farm suggested an initial introduction of the LP progenitor and a subsequent switch to HPAIV H7N7 after the incursion. The findings provide rare field evidence for a shift in pathogenicity of a notifiable AIV infection and re-inforce the validity of current approaches of control measures to curtail low pathogenic H5 and H7 virus circulation in poultry.

KEYWORDS
avian influenza, disease control, H7, molecular epidemiology, outbreak investigation, pathogenicity, quasispecies

*These authors contributed equally.

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1 | INTRODUCTION

Avian influenza virus (AIV) infections represent one of the most dominant and complex animal health threats in global poultry production. The viruses that are grouped within the genus *Orthomyxovirus* of the *Orthomyxoviridae* family are further classified into subtypes based on antigenic properties of the hemagglutinin (HA) and the neuraminidase (NA) surface glycoproteins. Besides the antigenic HA and NA classification, AIV can be classified according to their virulence in chicken into low pathogenic avian influenza viruses (LPAIV) and high pathogenic avian influenza viruses (HPAIV). The majority of known AIV is low pathogenic causing minimal or no adverse health effects in poulty. One of the decisive events at genome level for a shift in pathogenicity is the acquisition of basic amino acids at the hemagglutinin cleavage site (HACS). So far, only H5 and H7 AI viruses, but no other subtypes, have been observed to acquire such mutations under natural circumstances (Alexander, 2000). The emergence of such mutations, leading to the conversion of a trypsin-like protease cleavage site in LPAIV to a subtilisin-sensitive one in HPAIV, may result from substitution or insertion of purine nucleobases encoding basic amino acids lysine and/or arginine at the region of the cleavage site (CS) of the HA (Abdelwhab, Veits, & Mettenleiter, 2013) or from segmental insertional recombination with similar effects (Suarez et al., 2004). With respect to function, these mutations render the HACS accessible to subtilisin-like proteases and, hence, allow systemic viral replication. In contrast, replication of LPAIV expressing a trypsin-sensitive HACS is confined to epithelia of the respiratory and gastrointestinal tracts (Spackman, 2008).

In consequence, regardless of their actual pathogenicity, occurrence of viruses belonging to the subtypes H5 and H7 is notifiable and has to be addressed with control measures aiming at the reduction of disease spread and eventually to achieve eradication (OIE, 2015a). Within the European Union, control measures addressing notifiable AI (i.e., infections with subtypes H5 and H7) are defined in Council directive 2005/94/EC (Anonymous, 2005). While the control of HPAIV is indisputably justified by their detrimental impact on poultry production, the rationale for the stringent control of LPAIV cannot directly be associated with either an immediate impact on the poultry sector or potential zoonotic implications (with the sole exception of highly zoonotic LPAIV H7N9 strains in China (Gao et al., 2013)). Instead, it is based on the evidence that H5 and H7 LPAIV may represent progenitor viruses of HPAIV variants that result from spontaneous mutations of the former. LPAIV are widely circulating among aquatic wild bird species that form the natural reservoir for these viruses (Munster & Fouchier, 2009). Wild bird–livestock interactions may lead to spillovers of infections to domestic poultry, mainly ducks and geese in first instance. At last, after a phase of adaptation, stable and efficient replication may be established including the further adaptation to gallinaceous birds, which is believed to be a prerequisite to an increased risk of mutation to HPAIV variants (Campitelli et al., 2004; Lebarbenchon & Stallknecht, 2011; Verhagen et al., 2017).

Phylogenetic analyses of subtype H5 and H7 viruses revealed the development of geospatially confined lineages within LPAIV, but not HPAIV pathotypes, supporting the hypothesis of their evolution from single-specific LP progenitors (Abdelwhab, Veits, & Mettenleiter, 2014; Lebarbenchon & Stallknecht, 2011). Monne et al. (Monne et al., 2014) have shown the evolution of LPAI viruses within a larger epizootic including the emergence of an HPAI variant rooting in the LPAIV quasispecies cloud.

In 2015, four incursions of subtype H7N7 LPAIV into poultry as well as the de novo generation of H7 HPAI viruses in two cases have been reported in Europe: two outbreaks in Germany, one outbreak each in the Netherlands and in the United Kingdom (Figure 1). The Animal and Plant Health Agency of the United Kingdom (Anonymous, 2015) published details on an H7N7 HPAI outbreak, where a combination of epidemiological and molecular data led to the conclusion that the pathogenicity shift from LPAI to HPAI had happened directly on the HPAI-infected premises. While it was possible to detect evidence of LPAI infection through serological findings and LPAI genome detection in this particular case, no epidemiologically connected active LPAI infection was found. Outbreaks caused by infections with H7N8 LPAIV and a closely related H7N8 HPAIV variant during the same epidemic have also recently been described in the United States of America (Killian et al., 2016; Lee, Torchetti, Killian, & Swayne, 2017). Phylogenetic characterization suggested the HPAI outbreak resulted from spontaneous mutation of the circulating LPAI progenitor. However, in this case, details on the LPAIV incursion on the HPAI outbreak farm are missing. A further example of indications on matching pairs of H7N7 LPAI precursor and HPAI mutant viruses was reported in Spain in 2009-2010, where the progenitors of the HPAIV strains were simultaneously circulating in wild birds yet lacking a geographic linkage (Iglesias, Martinez, Munoz, de la Torre, & Sanchez-Vizcaino, 2010). These examples underline the challenges to gather complete epidemiological and molecular evidence, whereby narrowing the spatiotemporal window to the single epidemiological event of the actual mutation from the LPAI progenitor to an emerging HPAIV variant (Abbas et al., 2010; Banks et al., 2001; Pasick et al., 2005).

The here presented study allowed us to have a closer look at a LP-to-HP emergence event in chicken layer flocks in Lower Saxony, Germany, based on detailed epidemiological and molecular investigations initiated to control a HPAI outbreak.

2 | MATERIALS AND METHODS

2.1 | Epidemiological investigations and sample collection

Epidemiological investigations fulfilling the requirements of the Council Directive 2005/94/EC (Anonymous, 2005) were performed on two farms in the municipality of Herzlake in Lower Saxony, Germany, initiated in response to the notification of the local veterinary authorities of suspected AIV H7N7 infections in two-layer flocks.

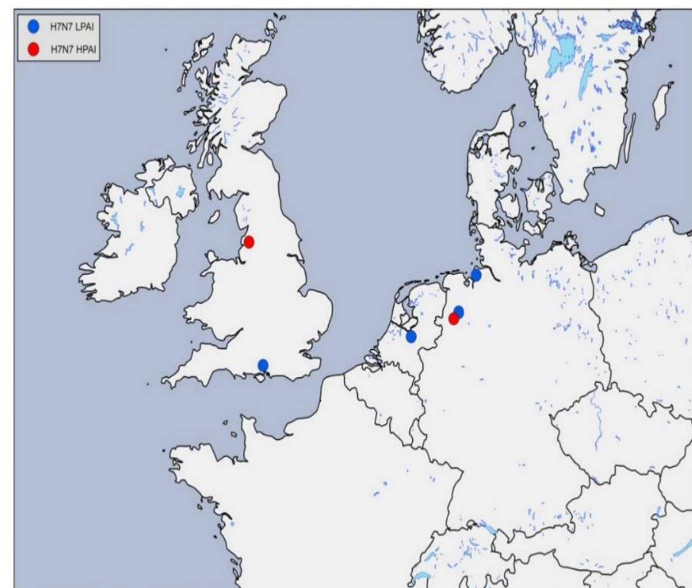

FIGURE 1 Geographic location of all notified H7N7 outbreaks in the European Union in 2015. Red dots signal HPAI outbreaks; blue dots depict LPAIV detection

Investigations aiming at the identification of potential sources of infection comprised of an on-farm assessment of the farming structure and structured interviews with the farm manager and the local veterinarian. Additional information was retrieved from production records, invoices and movement data of transport vehicles and livestock professionals visiting the holding. Samples for official disease confirmation were collected according to the Commission Decision 2006/437/EC (Anonymous, 2006) consisting of native oropharyngeal and cloacal swabs as well as serum samples. In addition, environmental samples were taken as part of detailed epidemiological follow-up investigations. An overview of the total numbers of samples is provided in the Supplemental Table S1. All farms located in the established restriction zones underwent examination to confirm freedom of AI by the veterinary authorities after zones had been established and before they were lifted.

2.2 | Detection, molecular sub- and pathotyping of AIV RNA

RNA was extracted from samples using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at −20°C until use. By real-time RT-PCR (RT-qPCR), presence of the influenza A virus matrix (M) gene was confirmed using primers and probes following the protocol of Hoffmann et al. (Hoffmann et al., 2010). AIV-positive samples were further examined by means of an H7- and H5-specific RT-qPCR, respectively (Hoffmann, Hoffmann, Henritzi, Beer, & Harder, 2016). While no evidence of H5-specific RNA was obtained, H7-positive samples were pathotyped using newly developed RT-qPCRs that allow rapid differentiation between LP and HP H7 virus strains by probe-assisted detection of the HA cleavage site; amplicons produced from these RNAs by H7-specific RT-qPCR analysis were further used for sequence analysis of the H7 HACS using the H7-specific reverse primer mix for Sanger sequencing (Graaf, Beer, & Harder, 2017). The RT-qPCR reactions were performed in 25 μl volumes using SuperScript® III One-Step RT-PCR kit with Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, USA) on a CFX96 thermocycler machine (Bio-Rad Laboratories, Hercules, USA).

2.3 | Virus isolation

Virus isolation from selected cloacal and tracheal swab samples that had tested positive for AIV subtype H7 in the RT-qPCR was performed in 10-day-old specific pathogen-free (SPF) chicken eggs according to standard protocols. After five days of incubation at 37°C, allantoic fluids were assayed for haemagglutinating activity (HA) (OIE, 2015b). The identities of sub- and pathotypes were confirmed by HACS sequence analysis using pan-HA and pan-NA RT-PCR assays as described by Gall et al. (Gall, Hoffmann, Harder, Grund, & Beer, 2008). Isolates were stored at −70°C until further use.

2.4 | *In vivo* pathotyping

Pathogenicity was determined by measuring the intravenous pathogenicity index (IVPI) test following the IVPI protocol of the OIE (OIE, 2015a). Ten specific pathogen-free (SPF) 6-week-old chickens were inoculated intravenously with 0.1 ml of the egg-passaged H7 isolates diluted 1:10 with phosphate-buffered saline (PBS). For 10 days, clinical signs were monitored. At each daily observation, every chicken was scored 0 (normal/healthy), 1 (sick), 2 (severely sick) or 3 (dead). The animal trials for the standardized in vivo pathotyping were approved by the State Office for Agriculture, Food Safety and Fisheries of Mecklenburg-Wester Pomerania, Rostock, Germany (reference number: LALLF M-V/TSD/7221.3-2.2-005/11).

2.5 | Detection of H7-specific antibodies by enzyme-linked immunosorbent assay (ELISA) and hemagglutination inhibition assay

After heat inactivation (30 min, 56°C), chicken sera obtained from both poultry holdings were tested to detect antibodies against the AIV nucleoprotein (NP) using a Influenza A Antibody Competition ELISA (ID Screen®, IDVET, Grabels, France) according to the manufacturer's instructions. To classify samples as positive or negative, the cut-off value recommended by the manufacturer was used: Percent inhibition (PI) values ≤55% were considered as positive, PI ≥ 65% as negative and PI between 55% and 65% as inconclusive.

To further classify and subtype AIV, haemagglutination inhibition assays using antigens of subtypes H5 and H7, including the homologous LPAIV and HPAIV H7N7 antigen, were performed according to the diagnostic manual of the European Union (Anonymous, 2006). Inhibition at a serum dilution of at least 1/16 was regarded as positive.

2.6 | Nucleotide sequencing analyses

2.6.1 | Sanger sequencing

Pathotyping was carried out by nucleotide sequence analysis of amplicons of the HACS (OIE/FAO, 2015). Amplicons were size-separated by agarose gel electrophoresis, purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and directly used for cycle sequencing reactions (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Darmstadt, Germany). The reaction products were purified using NucleoSEQ columns (Macherey–Nagel GmbH & Co., Berlin, Germany) and sequenced on an ABI PRISM® 3100 Genetic Analyzer (Life Technologies, Darmstadt, Germany). Obtained sequences were assembled and edited manually using the Geneious software, version 10.0.09 (http://www.geneious.com (Kearse et al., 2012)).

2.6.2 | NGS methodology

Undirected shotgun Next Generation Sequencing (NGS) from virus isolates was performed as previously described by Juozapaitis et al. (2014).

In brief, RNA of influenza samples was extracted using Trizol LS (ThermoFisher Scientific, Waltham, USA) and QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) and used as template for cDNA synthesis with cDNA Synthesis System REF 11 117 831 001 (Roche, Mannheim, Germany). Fragmentation of the cDNA was performed with Covaris M220 ultrasonicator (Covaris Ltd, Brighton, UK) applying a target size of 300 bp. The fragmented cDNA was then converted into barcoded libraries using Illumina compatible adapters (Bioo Scientific Corp., Austin, USA) using a SPRI-TE library system (Beckman Coulter, Brea, USA) with SPRIworks Fragment Library Cartridge II (for Roche FLX DNA sequencer; Beckman Coulter) without size selection. Upper and lower size selection was performed manually using Agencourt® AMPure® XP magnetic beads (Beckman Coulter). The libraries were then quality and quantity checked using a Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany) and Kapa Library Quantification Kit for Illumina (Kapa Biosystems, Cape Town, South Africa) on a Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, USA). Sequencing was performed with an Illumina MiSeq instrument with MiSeq reagent Kit v3 for 600 cycles (Illumina, San Diego, USA). Consensus sequences were generated using an iterative assembly and mapping approach performed with Newbler Genome Sequencer software (v. 3.0; Roche, Mannheim Germany) and Geneious software suite (v. 9.1.6; Biomatters, Auckland, New Zealand). The generated alignments were taken to determine minor variants using generic SNP finder of Geneious software suite with minimum variant frequency of 10%, applying parameters of maximum *p*-value of 10^{-5} and filter for strand bias. Consensus sequences were deposited in the EpiFlu™ database (platform.gisaid.org) under accession numbers EPI_ISL_191763-64 and EPI_ISL_191941-42. Deep sequencing information of the cleavage site was obtained by extracting RNA (see methodology described above) of a set of samples as template for generating amplicons gained with PCR primers containing IonTorrent barcodes using Invitrogen Superscript III One-Step RT-PCR (Thermo-Fisher Scientific, Waltham, USA).

(HLPAI_H7EU_P1_RV HLPAI cleavage site, CCTCTCTATGGGCAGTCGGTGATCCCATCCRTTTTCRATGAWTCC; HLPAI_H7EU_P1_FW, CCTCTCTATGGGCAGTCGGTGATGATGCTRGCAAMAGGRATGAA; HLPAI_H7EU_A_74_RV, CCATCTCATCCCTGCGTGTCTCCGACTCAGCGATCGGTTCGATCCCATCCRTTTTCRATGAWTCC; HLPAI_H7EU_A_73_FW, CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTGCCTGTCGATGATGCTRGCAAMAGGRATGAA; HLPAI_H7EU_A_70_RV, CCATCTCATCCCTGCGTGTCTCCGACTCAGCCTACTGGTCGATCCCATCCRTTTTCRATGAWTCC; HLPAI_H7EU_A_69_FW, CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCAATTGGCGATGATGCTRGCAAMAGGRATGAA). These libraries were then quality checked using a Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany) and Kapa Library Quantification Kit (Kapa Biosystems, Cape Town, South Africa) on a Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, USA) and subsequently sequenced on an IonTorrent PGM (Thermo Fisher, Waltham, USA) with Hi-Q sequencing reagents. Raw sequence data were quality trimmed and screened for adapter, barcodes and primer contamination. Sequences were size selected and reads between 30 and 60 bp, and thereby covering the cleavage site, were extracted. These datasets were assembled with generic mapper in Geneious software suite (v. 9.1.6; Biomatters, Auckland, New Zealand) with a stringency of 100%. Consensus sequences were generated and aligned. Consensus sequences were manually evaluated and trimmed to a unique length. These sequences served as template for stringent (100%) mapping of the original entire dataset. The contigs, covering 99% of the raw data, were annotated and the cleavage site classified as HPAI or LPAI, respectively.

2.7 | Phylogenetic and RNA structural analyses

Generated sequences were collected and edited using the Geneious software version 10.0.09 (Kearse et al., 2012). The sequences were

aligned with a selection of the closest related sequences (Eurasian lineage), as obtained by nucleotide megaBLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) together with other sequences selected as representatives for more distant clusters, like the Asian H7N9 lineage. Alignment and identity matrix analyses were afterwards performed using MAFFT version 7.017 (Katoh, Misawa, Kuma, & Miyata, 2002). For phylogenetic maximum likelihood analyses, the IQ-Tree software version 1.3.13 was used (Nguyen, Schmidt, von Haeseler, & Minh, 2015) using the TIM+Γ+G4 model according to the BIC ranking provided by the IQ-Tree. Resulting consensus trees were viewed, edited and annotated using FigTree v1.4.2 software (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape 0.48 (http://inkscape.org/en).

RNA secondary structures around the cleavage site encoding part of the HA genome segment were examined using the Quickfold (http://unafold.rna.albany.edu/?q=DINAMelt/Quickfold) and RNA fold programmes (http://rna.tbi.univie.ac.at/). Sequences of closely related H7 viruses from Germany, England and the Netherlands obtained during outbreaks in 2015 were selected for comparison.

3 | RESULTS

3.1 | Case description and epidemiological investigations

The initially affected farm (hereafter referred to as farm A) was a layer farm with 36,200 hens operating in an outdoor production system. The farm is located in the district of Emsland, Lower Saxony, in the vicinity to a river and surrounded by a prevailing watershed management of agricultural fields using open drainage, making the area attractive for wild water birds throughout the year.

A drop in egg production by 16% in the period between June 6 and 8, 2015, led to clinical inspection of the flock. No further clinical symptoms were developed. Samples taken revealed detection of AIV H7-specific RNA genome fragments in a private laboratory, and the outbreak suspicion was reported to the district veterinary office on June 9, 2015. A farm quarantine was implemented; official samples (40 combined oropharyngeal/cloacal swabs from 40 birds and 20 sera) were taken for outbreak confirmation; and epidemiological investigations were initiated. The national reference laboratory (NRL) for avian influenza confirmed on June 11, 2015, that the outbreak had been caused by LPAIV of subtype H7N7 (see laboratory results below). All chickens on the farm were culled and safely disposed. Active surveillance in the established restriction zones and investigations on farms with direct or indirect contact to the infected premises revealed no further outbreaks. Epidemiological tracing did not gather evidence for production-related inputs and services such as animal trade, feed, drinking water, the vehicles of suppliers and related factors as potential sources of infection. No linkages with poultry farms in the close vicinity were identified. The veterinary authorities concluded that contact to wild birds was the most likely source of infection and that the infection had most probably taken place 2 to 4 weeks prior to the first detection of clinical signs.

A second poultry farm (hereafter referred to as farm B) was located at a distance of approximately 400 metres from farm A. The overall setup of farm B, a commercial poultry holding keeping approximately 10,000 laying hens split in four different age groups, is depicted in Figure 2. The animals were kept in two stables (1 and 2) that housed two groups each (groups hereafter labelled 1a/1b and 2a/2b). The stables were connected by a building comprising the entry facility as well as the egg collection and packaging centre.

Farm B was first sampled during active surveillance within the restriction zone 4 days after initial clinical findings on farm A (combined oropharyngeal and cloacal swabs from 20 animals and additional 20 serum samples) and subsequently 4 weeks afterwards (combined oropharyngeal and cloacal swab samples from 20 animals). All samples, taken equally distributed over the four groups, tested negative on both occasions.

The first clinical signs were observed on farm B on July 23, 16 days after the second negative surveillance testing. Mortality

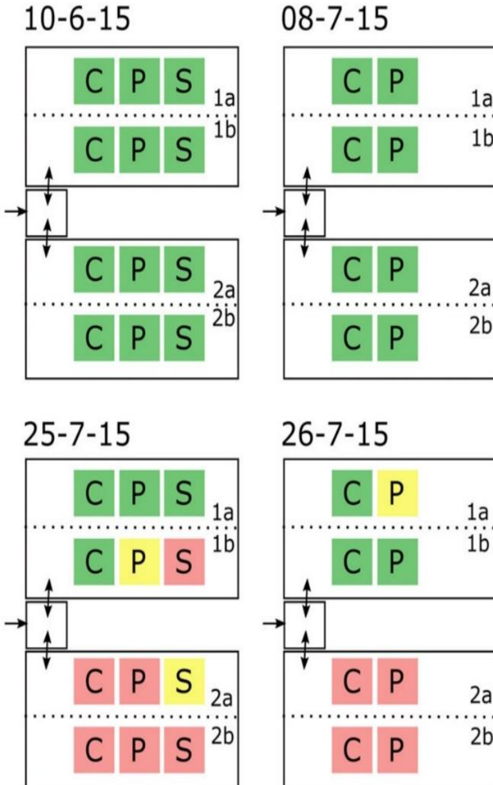

FIGURE 2 Sketch of farm B with two stables (1 and 2) consisting of two groups each (a and b) accessible through a connecting building with a hygiene lock (one-headed arrow). The status of the four layer flocks on the farm according to clinical presentation (C), virological investigations by PCR (P) and AIV-specific serology (S) is indicated for four different dates shown on top of each graph. Green colour signals absence of clinical signs, lack of detection of influenza RNA by PCR and lack of H7 seropositive chicken. Red colour indicates severe clinical signs and increased mortality, presence of animals with high viral loads of AIV H7 and detection of seropositive animals. Yellow colour stands for the presence of a few animals with low viral loads and inconclusive ELISA results

increased overnight, that is 50 (1.85%) hens were found dead in group 2b, housing 2706 animals at that time, comparing to a total of 19 (0.69%) dead animals for the entire time between June 1 and July 22, 2015, with a maximum of two dead hens per day. The dead birds initially clustered in the part of the pen closer to the entry door. In addition, a drop in egg production, recorded at farm level only, became evident (Figure 3). With the detection of H7-specific genome fragments by a private laboratory on July 24, 2015, the farm was quarantined and sampled for official disease confirmation (20 serum samples as well as combined oropharyngeal and cloacal swabs from 40 animals, samples taken evenly from the four groups). On July 25, 2015, a further increase in animal losses was recorded (additional 100 fatalities overnight in group 2b) and mortality started to rise in group 2a (10 fatalities overnight out of a total of 2916 hens). The presence of AIV H7N7 was confirmed by the NRL on July 26, 2015 (see laboratory results below). A synopsis of the observed development of clinical signs, serological and virological results for the different animal groups is depicted in Figure 2.

Epidemiological tracing did not find any indication that trade or otherwise production-chain-related contacts of the farm were the potential route of entry for the virus. The on-farm assessment identified two key breaches of biosecurity: First, the entrance for the farm workers into the stable units failed to allow separating clean and unclean clothes or equipment, but made cross-contamination and bypassing of hygiene measures possible. Second, the ventilation of the animal facilities was lacking a wild bird-proof grid on the supply side, thus allowing wild birds to stay under the roof of the building and to act as a potential source of infection through either contamination or direct contact. In addition, a possible interaction with wild birds was supported by the presence of maize silage heaps with open access to wild birds in close vicinity to the poultry facilities on the farm premises.

As part of the outbreak control measures, a surveillance zone with 10 km radius was established around farm B. A total of 226 poultry holdings identified in the zone underwent clinical and virological examination without positive findings throughout the event.

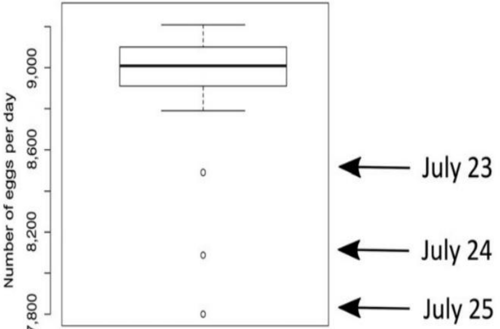

FIGURE 3 Box plot of daily egg production of farm B between July 1 and 25, 2015; outliers illustrate the onset of increased mortality starting on July 23, 2015

3.2 | Virological investigations and virus characterization

Of the 39 available swab samples obtained from farm A, 11 tested positive by the AIV matrix (M) gene RT-qPCR. Presence of subtype H7 viral RNA was confirmed by a H7-specific RT-qPCR in all M-positive samples. The NA subtype was determined as N7 by sequence analysis in selected samples with a high load of viral RNA (not shown). All swab samples obtained from farm B on Day 1 and Day 29 after start of the LPAI event (active surveillance) tested negative for AIV.

From farm B, a total of 105 swab samples were taken after the onset of clinical signs. In addition, samples from the environment of stable 2a/b were obtained. The synopsis of the M- and H7-specific RT-qPCR results is depicted in Figure 2. Eighteen swab samples of farm B tested positive for HP H7 and six different ones were positive for the LP variant by a pathotype-specific RT-qPCR (Graaf et al., 2017). One of the environmental samples taken in stable 2 (A/environment/Germany/AR1527/2015) contained genome fragments of both the HP and the LP variants (Table S1).

Sanger sequencing analysis of the HACS of samples originating from farm A revealed a monobasic motif (PEIPKGR*GLF). Samples from farm B that tested HP-positive in the pathotype-specific RT-qPCR revealed multiple basic amino acids at the cleavage site due to insertion of six nucleotides and a single transitional nucleotide substitution (G to A) (PEIPKRKRR*GLF) (see Figure 4). This confirms the molecular pathotyping results for LP and HP phenotypes obtained by RT-qPCRs. In addition to this, all obtained Sanger sequence data were identical to the later on established genome sequences based on NGS.

Furthermore, four selected swab samples with high viral loads of H7N7 according to the RT-qPCR were used for virus isolation (Table S1). The mean death time (MDT) of eggs inoculated with samples from farm A (A/chicken/Germany/AR915/2015 and A/chicken/Germany/AR942/2015) was >72 hours, while the MDT for farm B samples A/chicken/Germany/AR1385/2015 and A/chicken/Germany/AR1386/2015 was 48 hr. Pathogenicity testing in chickens of reference isolates AR915/2015 (farm A) and AR1385/2015 (farm B) revealed IVPI values of 0 and 2.8, respectively, confirming the molecular diagnosis of an LP and HP phenotype.

3.3 | Retrospective serological investigations

Of the 20 serum samples available from farm A, 15 tested positive in the influenza NP-specific ELISA. Antibody specificity was confirmed by HI assays using the homologous H7N7 antigens of isolates AR915/2015 (LPAI H7N7) and AR1385/2015 (HPAI H7N7). Thirteen sera from farm A showed specific HI titres ($\log_2$), which ranged between 5 and 10. None of the ELISA-positive sera reacted with H5 antigen (H5N1 A/chicken/Scotland/1959) in the HI assay (see Figure 5).

For farm B, seven of 20 serum samples, taken 46 days after onset of disease on farm A, showed positive results by ELISA and were tested with the same antigens as serum samples obtained from

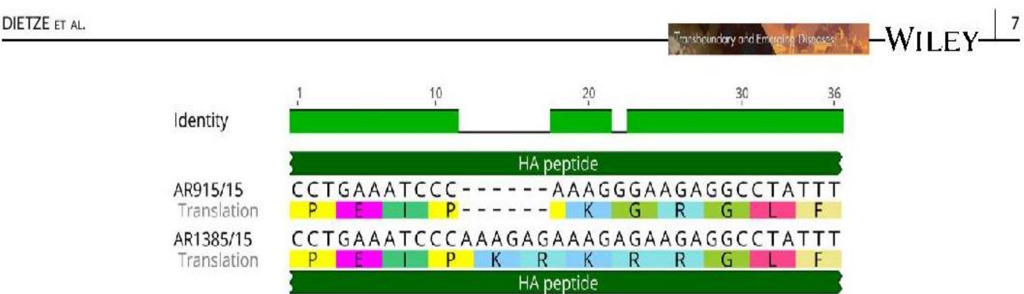

FIGURE 4 Sequencing results of the HACS of avian influenza subtype H7 LP (AR915/15) and HP (AR1385/15) viruses. Nucleotides in red indicate substitutional and insertional mutations

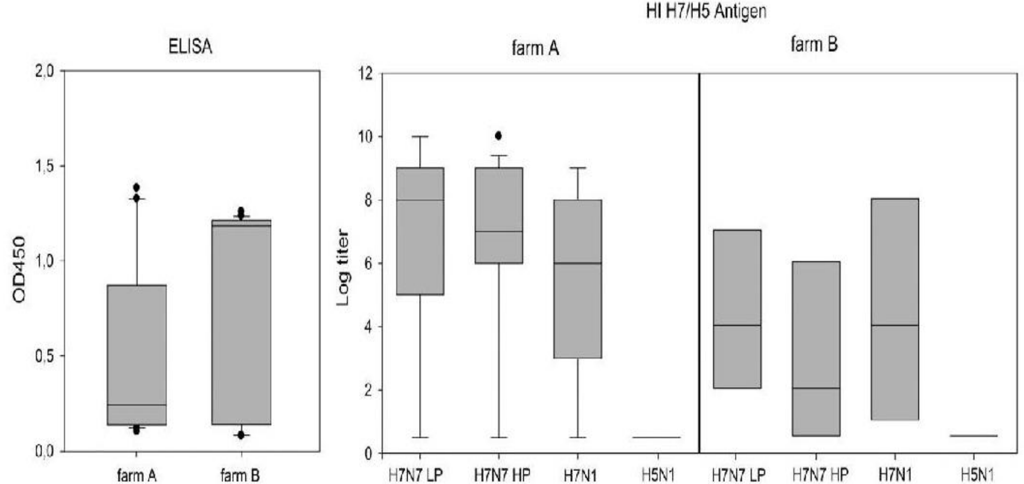

FIGURE 5 Results of serological investigations in farms A (LPAIV H7) and B (HPAIV) in Germany by competitive NP-specific ELISA and HI assays using the indicated antigens. HI titres are presented as the highest $\log_2$ dilution step showing hemagglutination inhibition

farm A. Five of these ELISA-positive sera showed specific HI titres, which ranged between 4 and 8. None of the ELISA-positive sera reacted with H5 antigen (H5N1 A/chicken/Scotland/1959) in the HI assay.

3.4 | Nucleotide sequence characterization and phylogenetic analysis

The genome of four representative influenza A virus isolates originating from the LP/HPAI H7N7 outbreaks in the two-layer farms was sequenced. Molecular analysis of the four isolates confirmed the presence of an HP strain in two samples derived from farm B (insertion of the motif PEIPKRKRR*GLF at the HA cleavage site) and identified two isolates of farm A as LP H7N7 viruses (PEIPKGR*GLF). In addition to the cleavage site, two further nucleotide substitutions were found in the HA gene distinguishing the LP and HP virus isolates (Table 1). This included an amino acid change at position I13S of the HA protein, resulting in a homology of 99.5% between the HA proteins of the LP and the HP viruses. Undirected shotgun Next Generation Sequencing (NGS) of the HA segment of LPAIV AR915/15 with mean coverages of 403 reads showed no evidence for the presence of any insertions in the HACS as minor variants. Likewise, NGS sequencing of barcoded amplicons of the HA cleavage site of independent swab samples from farm A (AR915/15; AR942/15), yielding high sequencing depth with coverages of 85.606 to 234.258 reads, gave no indications for HP HACS sequences. In contrast, NGS sequencing of amplicons of the HACS from swab samples collected from farm B (AR1527/15, AR1421/15, AR1546/15) showed that the samples represented a mixture of LP and HP cleavage sites. Moreover, the two analysed HP isolates differed from the LP H7 viruses by mutations in the PB2, PB1, PA, NP, NA, M and NS genes. In each of the PB2, PB1, NP, NA, M2 and NS1 proteins, nonsynonymous mutations were deduced (Table 1). None of these mutations have been assigned to any specific functions, although the mutation N92D in NS-1 affected a position that has been associated with interferon escape (Ngunjiri et al., 2013). Five of these coding mutations were already present as minor variants in the putative LP H7 progenitor virus AR915/15 showing significant frequencies from 18% to 42% (Table 1).

For the further phylogenetic characterization, the HA nucleotide sequences of the isolates were utilized to conduct a megaBLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi; accession date August 1, 2017) in the database "Nucleotide collection (nr/nt)" to identify closely related sequences. Highest identities at the nucleotide level were observed for AIV from the Netherlands (e.g., A/chicken/Netherlands-Barneveld/15004745/2015; H7N7 LP) and the United Kingdom (A/chicken/England/26352/2015; H7N7 HP) originating from an outbreak in 2015. To refine these findings, phylogenetic

TABLE 1 Mutations identified outside the hemagglutinin cleavage site in the highly pathogenic H7N7 avian influenza virus AR1385/15 in comparison with its low pathogenic AIV AR915/15 precursor isolated from outbreaks in the district of Emsland, Lower Saxony, Germany, in 2015

Gene segment	Mutations in AR1385/15		Mutations as minor variants in AR915/15	
	Silent	Coding	Silent	Coding
PB2	2	E123K, I147V, K355R	1 (21.3%)	E123K (22.6%), K355R (42.2%)
PB1	2	F254C	0	F254C (18.8%)
PA	3	K185R	0	K185R (18.2%)
HA	1	I13S	0	0
NP	1	S478F	0	0
NA	3	V439A	1 (23.8%)	0
M	2	V68L (M2)	1 (19.7%)	0
NS-1	0	N92D	0	N92D (25.1%)

analyses were conducted using a maximum likelihood approach (IQ-Tree) based on the TIM+ Γ +G4 model (Nguyen et al., 2015). As shown in Figure 6, the four virus isolates from the LP and HP outbreaks in Germany clustered with the Eurasian H7 lineage. They formed a distinct group defined by high bootstrap values (100%) and long branches in close relation to viruses of contemporary outbreaks in England and the Netherlands in 2015, but were clearly distinct from H7 viruses of the highly zoonotic Chinese lineage. Another H7N7 outbreak in a second holding in the UK in 2015 was phylogenetically unrelated to the cases in Germany, the Netherlands and the first case in the UK.

To further describe the relationship between the H7N7 viruses from the UK and Germany, concatenated whole-genomes of these viruses were compared. This analysis substantiated the close relationship of the isolates, but indicated also that the UK viruses were clearly different, as they contained NS gene segments with only 72.1% identity in the genome sequence indicating their origin in distinct alleles (Figure S1).

3.5 | RNA secondary structure analysis

A stretch of RNA nucleotides encoding the HACS was predicted to fold into a small stem-loop structure for both the LP and HP variants of the H7N7 viruses from Germany (Figure 7). For the LP sequence (AR915/15), the loop included the A- and G-rich part, which encoded the HA1-C-terminal arginine and the glycine forming the new N-terminus of the HA2 protein part after endoproteolytical processing. The closely related LP H7 Dutch and German viruses revealed the same HACS-encoding nucleotide sequence and, hence, secondary structural prediction as shown for AR915/15. This is also true for another German LPAI virus, detected in an unrelated outbreak in a turkey holding in Cuxhaven, Germany, in March 2015 (not shown). A similar structure was also predicted for the sequence of the HP phenotype (AR1385/125), where the inserted untemplated A and G residues grouped into a larger terminal bulge.

4 | DISCUSSION

In 2015, AIV H7N7 outbreaks were reported in the European Union from Germany, the United Kingdom and the Netherlands (Figure 1). In-depth epidemiological investigations and genetic characterization of the two separately reported but spatiotemporally linked H7N7 outbreaks in Germany provided evidence of a very close evolutionary relationship of the involved viruses despite their difference in pathogenicity, that is they form a matching pair of progenitor LP and inheritor HP viruses.

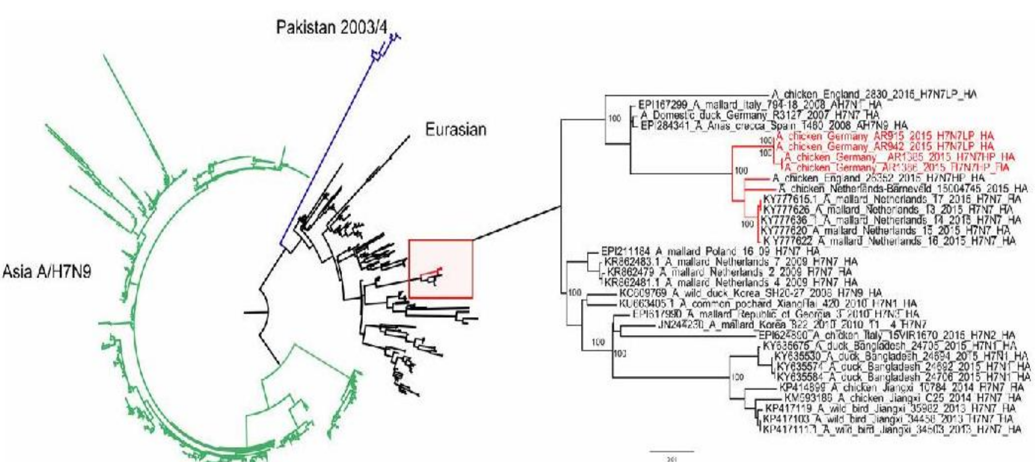

FIGURE 6 Phylogenetic tree of the hemagglutinin (HA) gene (full-length coding sequence) of low and highly pathogenic avian influenza viruses (LP/HPAIV) of subtype H7 viruses derived from poultry and wild birds in Eurasia in the period from 1999 to 2015 (a). The zoomed section (b) depicts the topology of viruses detected in two chicken layer farms in Germany, 2015 (red virus designations). Phylogenetic analysis was conducted using a maximum likelihood approach (IQ-tree) based on the TIM+I+G4 model (Nguyen et al., 2015)

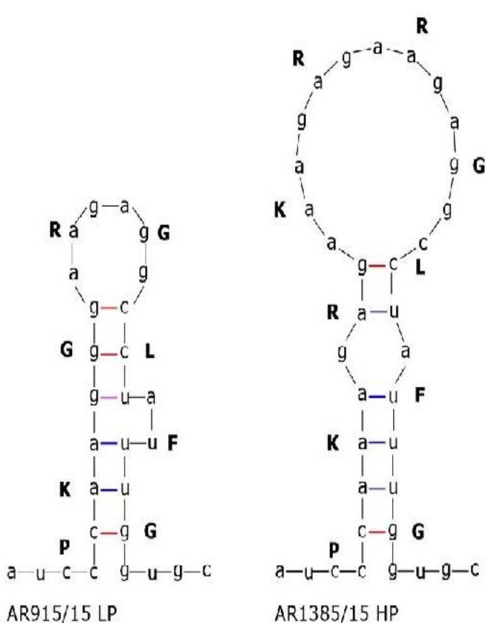

FIGURE 7 Comparison of secondary RNA structures of sequences encoding the hemagglutinin cleavage site of the LP (AR915/15, left) and HP (AR1385/15, right) H7N7 AIV originating from outbreaks in Germany in 2015. RNA secondary structures were generated by the Quickfold programme. Coloured lines are depicted as follow: blue—double hydrogen bond; red—triple hydrogen bond; pink-wobble-base pair

With a remaining level of uncertainty that is unavoidable in field investigations, the course of the epidemiological event is postulated as follows. Farm A became infected with LPAIV H7N7, probably through the wildlife—domestic bird interface. The incursion occurred most likely several weeks before the infection was confirmed on June 11, 2015. Until completion of the cleansing and disinfection of the premises on June 16, 2015, farm A must be considered a possible source of LPAIV infection for the surrounding area, including farm B. Identifying the time point and source of LPAIV infection of fram B is difficult given the small numbers of samples taken here before the outbreak of HPAI was suspected on 23 of July. The available limited laboratory results would suggest that farm B got infected with LPAI H7 after July 8, 2015, as animals of the farm tested negative for AIV on this day. This would bring the postulated time of LPAI infection fairly close to an anticipated time of the shift from LPAI to HPAI, as about two weeks are assumed to pass before clinical manifestation of HPAI becomes evident (Bos et al., 2007). It has to be taken into account, however, that the approach of the competent veterinary authority to consider farm B as a single epidemiological unit despite its separation in four compartments, with reduced animal contacts and interactions between compartments (Figure 2), may have significantly reduced the overall likelihood of LPAIV detection, in particular at an early stage of infection. On basis of the reported five animals that were tested per group, the detectable prevalence was only 50% at the 95% confidence level. Therefore, the introduction of LPAI on farm B before July 8, 2015, cannot be excluded. This re-emphasizes the challenges in early LPAI detection due to nonadapted sampling frames (Gonzales, Boender, Elbers, Stegeman, & de Koeijer, 2014) and, as expected from previous experience with LP H7N7 incursions, the lack of clinical manifestations allowing early detection (Probst et al., 2012). Gaps identified in the biosecurity management of farm B, which operated in a closed indoor production system, led to several hypotheses of how virus-contaminated material may have entered the stables. The introduction likely occurred in stable 2, where both groups harboured seropositive animals, whereas in stable 1, group 1a tested negative throughout the event.

Limitations of further sampling for the present study resulted from pressing needs for immediate destruction and safe disposal of animals, which was given highest priority by the veterinary authorities. Nevertheless, a series of factors strongly suggested that initially farm B was infected by LP AIV H7 and that the subsequent HP mutation event took place there. These factors entail the epidemiological context, the close genetic relationship between the two variants and the detection of both LP and HP specific genome fragments in an environmental sample of farm B. In addition to that, we observed the presence of mutations found in HP strains as minor variants in LP strains from farm A, and the seroconversion in parts of the chicken population of farm B; chickens following exposure to replication-competent HPAIV are expected to die before they seroconvert. It is interesting to note that seropositive birds could be detected not only within the group where clinical (HP) symptoms were first observed (2b), but also within two other groups (2a and 1b), indicating within-farm spread of LPAIV H7 to at least three groups. Furthermore, the observation that only part of the animals were AIV-H7 seropositive points to an early stage of LPAI virus circulation and a rapid LP-to-HP mutation, leaving a substantial number of animals susceptible to HPAIV H7 infection. An immune response to the antigenetically homologous/identical LPAI virus would have been able to protect animals from HP AIV-induced clinical symptoms (Fereidouni et al., 2009; Vergara-Alert et al., 2013). It is therefore reasonable to speculate that the emergence of HPAI requires triggering by the LP-to-HP pathogenicity conversion event itself, but also depends on the favourable competition and dynamic of spread of the HP variant within an at least partially susceptible host population.

Intensive surveillance in the vicinity of the affected farms gave no further indications for AIV circulation in either poultry or wild birds during the events. AIV H7 had not been detected in wild birds in the wider region of the federal state of Lower Saxony throughout 2015. Of 840 wild birds tested in this period, only two samples tested positive for AIV, one was positive for H5 (no further subtyping performed) and one positive for not further specified subtypes other than H5 or H7 (Wildvogelmonitoring-Datenbank (restricted access): https://ai-db.fli.de accessed July 4, 2017). Therefore, independent circulation of the two phenotypes outside farm B and separate introduction events of the LP and the HP variants within a short period appear highly unlikely. However, our analyses do not

give decisive evidence about the mode of LPAI virus transmission from farm A to farm B. Possible continued replication of LPAIV in undetected avian carriers presumably within the same wild bird population introducing LPAIV H7N7 into farm A before entering farm B as well as unnoticed direct farm-to-farm transmission cannot be excluded.

Approximately at the same time of these events, an HPAI H7N7 outbreak was reported from the UK. The presence of H7 seropositive poultry on the affected farm in the UK indicated previous circulation of a potential LP progenitor, which, however, could not be retrieved. The H7N7 outbreak in the Netherlands in summer 2015 remained LP. Genetically, the HA of these viruses appeared to be closely related (Figure 6). It is interesting to note that viruses of this lineage apparently had a higher propensity to mutate to the HP pathotype as this happened at two independent outbreaks in the UK and in Germany. With the exception of the NS segment, all internal genome segments of these viruses were very closely related. It is unfortunate that, no full genome sequences were available from the contemporary Dutch isolate.

In Germany, an additional isolated outbreak of LPAIV H7N7 was confirmed in March 2015 in a commercial turkey holding. No genetic information apart from the HACS sequence is available for this case. There is no indication regarding an epidemiological link between this and the layer farms outbreaks.

The availability of a matching pair of H7 LP progenitor and HP inheritor viruses derived from the field allows further insights into potential prerequisites for the acquisition of an HP phenotype. Presence of a polybasic HACS has been identified as important in this respect, although several exceptions exist (Londt, Banks, & Alexander, 2007). A stuttering mechanism of the viral replication complex when copying the sequence encoding the LP cleavage site during antigenome and/or genome replication is believed to be at the basis of the insertional mutations of untemplated A and G residues, which finally translate into a furin-sensitive cleavage site. A recent observation indicated that a small stem-loop structure involving the HACS-encoding site might play a role in fostering stuttering replication activity (Nao et al., 2017). We were therefore interested to study the presence or absence of such stem loops in the investigated pair of LP and HP H7N7 viruses. A similar structure was identified in the H7 LP viruses studied here (Figure 7). However, no differences were seen between LP viruses (i.e., LP AR915/15) that eventually yielded an HP mutant (i.e., HP AR1385/15) or did not (Dutch H7 sequences of 2015). Therefore, it remains to be determined whether the presence or absence of such stem loops can be used to predict and quantify the risk of insertional mutations at this site. A number of previous studies have shown that the furin-sensitive cleavage site motif of the HA protein plays a major, yet not the sole, role in the conversion of phenotypes (Abdelwhab et al., 2016; Bottcher-Friebertshauser, Garten, Matrosovich, & Klenk, 2014; Gohrbandt et al., 2011; Munster et al., 2010). Several attempts failed to convert an LP virus into the HP phenotype by merely grafting, by reverse genetics, a polybasic HACS into the HA protein of an LP H5 or H7 virus (Schrauwen et al., 2011; Stech et al., 2009). These investigations gave evidence that further, as yet ill-defined, arrangements in either the HA or other viral proteins are required, at least in some viruses, in addition to a polybasic HACS (Abdelwhab et al., 2013; Diederich et al., 2015; Soda et al., 2011). Scattered throughout the genome of the currently studied LP/HP pair, ten amino acid substitutions outside the HACS were detected in the consensus sequence of the HPAI virus isolates, which allowed distinguishing the HP inheritor from its LP progenitor virus (Table 1). Five of them are already present as minor variants in the LP progenitor quasispecies, pointing to a certain importance of the whole virus population for the evolution of inner segments in the context of a LP-to-HP conversion. With the exception of mutation N92D in NS-1, none of the ten nonsynonymous mutations has been associated so far with specific functions, and none of them had been encountered in a study conducted by Maruyama et al. (Maruyama, Okamatsu, Soda, Sakoda, & Kida, 2013) in which conversion of an LP H7N7 to HP was achieved after grafting a polybasic HACS and consecutive serial passaging in chickens. In line with their conclusions, the actual contribution of one or more of the mutations outside the HACS to the generation of the HP phenotype of AR1385/15 remains indistinct and requires careful studying by reverse genetic technology. The presence of a matching set of mutations as minor variants in the LP progenitor with contemporaneous absence of any variants in the HACS also supports the assumption that LP-to-HP conversion is a progressive process. A similar observation has been made for the emergence of HP H7N1 from LP precursor viruses preceding the 1999/2001 HPAI epizootic in Italy (Monne et al., 2014). At last, the emergence of an HPAI phenotype might require a combination of a selection of variants of the inner segments already present in the quasispecies population and a subsequent shift in the HACS triggered by a stuttering virus replication fostered by RNA secondary structure.

In conclusion, the detection in the field of a HPAI virus causally and epidemiologically linked to an immediate LP progenitor virus remains a very rare event. However, this causal relation is fundamental for vindicating current LPAI control measures in domestic poultry. The presented findings, resulting from virological and close-meshed epidemiological investigations as part of AI control, describe such a rarely found spatiotemporally link as evidence of in vivo transformation of LPAIV to HPAIV within a single epidemiological event. It is recommended for future scenarios, to improve sampling frames in AI surveillance and control, taking a stricter definition of epidemiological units into account to facilitate the deeper understanding of outbreak dynamics, such as spread within and between farms, at better statistical power.

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CONFLICT OF INTEREST

None.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplemental material

Table: Results of real time RT-PCR (RT-qPCR) investigation using RT-qPCRs specific for a generic target (M), for subtype H7 (H7), and for the LP and HP pathotypes of H7 (H7 HP, H7 LP) in farms A (LPAIV H7) and B (HPAIV).

(A) Swab samples

	9 June 2015			Real-time RT-PCR			
		Farm	Stable	M PCR	H7	H7 HP	H7 LP
1	A/ck/Ger/AR909/2015	A	N/A	neg	neg	N/A	N/A
2	A/ck/Ger/AR910/2015	A	N/A	neg	neg	N/A	N/A
3	A/ck/Ger/AR911/2015	A	N/A	neg	neg	N/A	N/A
4	A/ck/Ger/AR912/2015	A	N/A	neg	neg	N/A	N/A
5	A/ck/Ger/AR913/2015	A	N/A	neg	neg	N/A	N/A
6	A/ck/Ger/AR914/2015	A	N/A	30.94	37.5	N/A	36.5
7	A/ck/Ger/AR915/2015	A	N/A	29.1	31.12	neg	33.38
8	A/ck/Ger/AR916/2015	A	N/A	neg	neg	N/A	N/A
9	A/ck/Ger/AR917/2015	A	N/A	32.99	33.4	N/A	33.8
10	A/ck/Ger/AR918/2015	A	N/A	neg	neg	N/A	N/A
11	A/ck/Ger/AR919/2015	A	N/A	neg	neg	N/A	N/A
12	A/ck/Ger/AR920/2015	A	N/A	neg	neg	N/A	N/A
13	A/ck/Ger/AR921/2015	A	N/A	neg	neg	N/A	N/A
14	A/ck/Ger/AR922/2015	A	N/A	neg	neg	N/A	N/A
15	A/ck/Ger/AR923/2015	A	N/A	neg	neg	N/A	N/A
16	A/ck/Ger/AR924/2015	A	N/A	neg	neg	N/A	N/A
17	A/ck/Ger/AR925/2015	A	N/A	neg	neg	N/A	N/A
18	A/ck/Ger/AR926/2015	A	N/A	neg	neg	N/A	N/A
19	A/ck/Ger/AR927/2015	A	N/A	neg	neg	N/A	N/A
20	A/ck/Ger/AR928/2015	A	N/A	neg	neg	N/A	N/A
21	A/ck/Ger/AR929/2015	A	N/A	39.79	38.8	N/A	35.66
22	A/ck/Ger/AR930/2015	A	N/A	39.85	39.35	N/A	35.35
23	A/ck/Ger/AR931/2015	A	N/A	neg	neg	N/A	N/A
24	A/ck/Ger/AR932/2015	A	N/A	neg	neg	N/A	N/A
25	A/ck/Ger/AR933/2015	A	N/A	neg	neg	N/A	N/A
26	A/ck/Ger/AR934/2015	A	N/A	38.58	35.56	N/A	neg
27	A/ck/Ger/AR935/2015	A	N/A	neg	neg	N/A	N/A
28	A/ck/Ger/AR936/2015	A	N/A	neg	neg	N/A	N/A
29	A/ck/Ger/AR937/2015	A	N/A	neg	neg	N/A	N/A
30	A/ck/Ger/AR938/2015	A	N/A	neg	neg	N/A	N/A
31	A/ck/Ger/AR939/2015	A	N/A	neg	neg	N/A	N/A
32	A/ck/Ger/AR940/2015	A	N/A	neg	neg	N/A	N/A
33	A/ck/Ger/AR941/2015	A	N/A	neg	neg	N/A	N/A
34	A/ck/Ger/AR942/2015	A	N/A	32.01	29.84	neg	28.89
35	A/ck/Ger/AR943/2015	A	N/A	36.88	34.54	neg	34.17
36	A/ck/Ger/AR944/2015	A	N/A	37.22	34.29	neg	35.75
37	A/ck/Ger/AR945/2015	A	N/A	39.15	38.04	neg	35.08
38	A/ck/Ger/AR946/2015	A	N/A	38.59	34.92	neg	35.17
39	A/ck/Ger/AR947/2015	A	N/A	neg	neg	N/A	N/A

	25 July 2015			Real-time RT-PCR			
		Farm	Stable	M PCR	H7	H7 HP	H7 LP
40	A/ck/Ger/AR1382/2015	B	N/A	30.06	36.22	33.48	neg
41	A/ck/Ger/AR1383/2015	B	N/A	27.95	32.95	32.33	neg
42	A/ck/Ger/AR1384/2015	B	N/A	31.03	36.48	36.6	neg
43	A/ck/Ger/AR1385/2015	B	N/A	18.76	27.03	19.01	neg
44	A/ck/Ger/AR1386/2015	B	N/A	24.29	28.05	17.34	neg
45	A/ck/Ger/AR1387/2015	B	N/A	neg	N/A	N/A	N/A
46	A/ck/Ger/AR1388/2015	B	N/A	neg	N/A	N/A	N/A
47	A/ck/Ger/AR1389/2015	B	N/A	neg	N/A	N/A	N/A
48	A/ck/Ger/AR1390/2015	B	N/A	neg	N/A	N/A	N/A
49	A/ck/Ger/AR1391/2015	B	N/A	neg	N/A	N/A	N/A
50	A/ck/Ger/AR1392/2015	B	N/A	neg	N/A	N/A	N/A
51	A/ck/Ger/AR1393/2015	B	N/A	neg	N/A	N/A	N/A
52	A/ck/Ger/AR1394/2015	B	N/A	neg	N/A	N/A	N/A
53	A/ck/Ger/AR1395/2015	B	N/A	neg	N/A	N/A	N/A
54	A/ck/Ger/AR1396/2015	B	N/A	neg	N/A	N/A	N/A
55	A/ck/Ger/AR1397/2015	B	N/A	neg	N/A	N/A	N/A
56	A/ck/Ger/AR1398/2015	B	N/A	neg	N/A	N/A	N/A
57	A/ck/Ger/AR1399/2015	B	N/A	neg	N/A	N/A	N/A
58	A/ck/Ger/AR1400/2015	B	N/A	neg	N/A	N/A	N/A
59	A/ck/Ger/AR1401/2015	B	N/A	neg	N/A	N/A	N/A
60	A/ck/Ger/AR1402/2015	B	N/A	neg	N/A	N/A	N/A
61	A/ck/Ger/AR1403/2015	B	N/A	neg	N/A	N/A	N/A
62	A/ck/Ger/AR1404/2015	B	N/A	neg	N/A	N/A	N/A
63	A/ck/Ger/AR1405/2015	B	N/A	neg	N/A	N/A	N/A
64	A/ck/Ger/AR1406/2015	B	N/A	neg	N/A	N/A	N/A
65	A/ck/Ger/AR1407/2015	B	N/A	neg	N/A	N/A	N/A
66	A/ck/Ger/AR1408/2015	B	N/A	37.98	N/A	neg	33.48
67	A/ck/Ger/AR1409/2015	B	N/A	38.37	N/A	neg	32.33
68	A/ck/Ger/AR1410/2015	B	N/A	neg	N/A	N/A	N/A
69	A/ck/Ger/AR1411/2015	B	N/A	38.52	N/A	neg	36.6
70	A/ck/Ger/AR1412/2015	B	N/A	38.33	N/A	neg	36.89
71	A/ck/Ger/AR1413/2015	B	N/A	36.77	neg	37.58	neg
72	A/ck/Ger/AR1414/2015	B	N/A	neg	N/A	N/A	N/A
73	A/ck/Ger/AR1415/2015	B	N/A	neg	N/A	N/A	N/A
74	A/ck/Ger/AR1416/2015	B	N/A	30.12	neg	30.69	neg
75	A/ck/Ger/AR1417/2015	B	N/A	neg	N/A	N/A	N/A
76	A/ck/Ger/AR1418/2015	B	N/A	neg	N/A	N/A	N/A
77	A/ck/Ger/AR1419/2015	B	N/A	neg	N/A	N/A	N/A
78	A/ck/Ger/AR1420/2015	B	N/A	neg	N/A	N/A	N/A
79	A/ck/Ger/AR1421/2015	B	N/A	29.88	36.23	31.14	neg
80	A/ck/Ger/AR1422/2015	B	N/A	neg	N/A	N/A	N/A
81	A/ck/Ger/AR1423/2015	B	N/A	neg	N/A	N/A	N/A
82	A/ck/Ger/AR1424/2015	B	N/A	neg	N/A	N/A	N/A
83	A/ck/Ger/AR1425/2015	B	N/A	neg	N/A	N/A	N/A
84	A/ck/Ger/AR1426/2015	B	N/A	neg	N/A	N/A	N/A
85	A/ck/Ger/AR1427/2015	B	N/A	neg	N/A	N/A	N/A
86	A/ck/Ger/AR1428/2015	B	N/A	neg	N/A	N/A	N/A
87	A/ck/Ger/AR1429/2015	B	N/A	neg	N/A	N/A	N/A
88	A/ck/Ger/AR1430/2015	B	N/A	neg	N/A	N/A	N/A
89	A/ck/Ger/AR1431/2015	B	N/A	neg	N/A	N/A	N/A
90	A/ck/Ger/AR1432/2015	B	N/A	neg	N/A	N/A	N/A

	25 July 2015			Real-time RT-PCR			
		Farm	Stable	M PCR	H7	H7 HP	H7 LP
91	A/ck/Ger/AR1433/2015	B	N/A	neg	N/A	N/A	N/A
92	A/ck/Ger/AR1434/2015	B	N/A	neg	N/A	N/A	N/A
93	A/ck/Ger/AR1435/2015	B	N/A	neg	N/A	N/A	N/A
94	A/ck/Ger/AR1436/2015	B	N/A	neg	N/A	N/A	N/A
95	A/ck/Ger/AR1437/2015	B	N/A	neg	N/A	N/A	N/A
96	A/ck/Ger/AR1438/2015	B	N/A	neg	N/A	N/A	N/A
97	A/ck/Ger/AR1439/2015	B	N/A	neg	N/A	N/A	N/A
98	A/ck/Ger/AR1440/2015	B	N/A	neg	N/A	N/A	N/A
99	A/ck/Ger/AR1441/2015	B	N/A	neg	N/A	N/A	N/A
100	A/ck/Ger/AR1442/2015	B	N/A	neg	N/A	N/A	N/A
101	A/ck/Ger/AR1443/2015	B	N/A	neg	N/A	N/A	N/A
102	A/ck/Ger/AR1444/2015	B	N/A	neg	N/A	N/A	N/A
103	A/ck/Ger/AR1445/2015	B	N/A	neg	N/A	N/A	N/A
104	A/ck/Ger/AR1446/2015	B	N/A	neg	N/A	N/A	N/A
105	A/ck/Ger/AR1447/2015	B	N/A	neg	N/A	N/A	N/A
106	A/ck/Ger/AR1448/2015	B	N/A	neg	N/A	N/A	N/A
107	A/ck/Ger/AR1449/2015	B	N/A	neg	N/A	N/A	N/A
108	A/ck/Ger/AR1450/2015	B	N/A	neg	N/A	N/A	N/A
109	A/ck/Ger/AR1451/2015	B	N/A	neg	N/A	N/A	N/A
110	A/ck/Ger/AR1452/2015	B	N/A	neg	N/A	N/A	N/A
111	A/ck/Ger/AR1453/2015	B	N/A	neg	N/A	N/A	N/A
112	A/ck/Ger/AR1454/2015	B	N/A	neg	N/A	N/A	N/A
113	A/ck/Ger/AR1455/2015	B	N/A	neg	N/A	N/A	N/A
114	A/ck/Ger/AR1456/2015	B	N/A	neg	N/A	N/A	N/A
115	A/ck/Ger/AR1457/2015	B	N/A	neg	N/A	N/A	N/A
116	A/ck/Ger/AR1458/2015	B	N/A	38.59	N/A	neg	neg
117	A/ck/Ger/AR1459/2015	B	N/A	neg	N/A	N/A	N/A
118	A/ck/Ger/AR1460/2015	B	N/A	neg	N/A	N/A	N/A
119	A/ck/Ger/AR1461/2015	B	N/A	neg	N/A	N/A	N/A
120	A/ck/Ger/AR1462/2015	B	N/A	26.28	33.92	27.43	neg

	30 July 2015			Real-time RT-PCR			
		Farm	Stable	M PCR	H7	H7 HP	H7 LP
121	A/ck/Ger/AR1536/2015	B	2b	31.17	38.14	32.17	neg
122	A/ck/Ger/AR1537/2015	B	2b	32.1	neg	33.75	N/A
123	A/ck/Ger/AR1538/2015	B	2b	neg	neg	n.d.	neg
124	A/ck/Ger/AR1539/2015	B	2b	31.04	38.76	31.4	neg
125	A/ck/Ger/AR1540/2015	B	2b	33.49	neg	34.46	neg
126	A/ck/Ger/AR1541/2015	B	2b	31.87	neg	33.18	neg
127	A/ck/Ger/AR1542/2015	B	2a	neg	neg	neg	N/A
128	A/ck/Ger/AR1543/2015	B	2a	neg	neg	N/A	N/A
129	A/ck/Ger/AR1544/2015	B	2a	neg	neg	N/A	N/A
130	A/ck/Ger/AR1545/2015	B	2a	neg	neg	N/A	neg
131	A/ck/Ger/AR1546/2015	B	2a	43008	34.7	31.46	N/A
132	A/ck/Ger/AR1547/2015	B	2a	neg	neg	N/A	N/A
133	A/ck/Ger/AR1548/2015	B	1b	neg	neg	N/A	N/A
134	A/ck/Ger/AR1549/2015	B	1b	neg	neg	N/A	N/A
135	A/ck/Ger/AR1550/2015	B	1b	neg	neg	N/A	N/A
136	A/ck/Ger/AR1551/2015	B	1b	neg	neg	N/A	N/A
137	A/ck/Ger/AR1552/2015	B	1b	neg	neg	N/A	N/A
138	A/ck/Ger/AR1553/2015	B	1b	neg	neg	N/A	N/A
139	A/ck/Ger/AR1554/2015	B	1a	neg	neg	N/A	N/A
140	A/ck/Ger/AR1555/2015	B	1a	neg	neg	N/A	N/A
141	A/ck/Ger/AR1556/2015	B	1a	neg	neg	N/A	N/A
142	A/ck/Ger/AR1557/2015	B	1a	neg	neg	N/A	N/A
143	A/ck/Ger/AR1558/2015	B	1a	neg	neg	N/A	N/A
144	A/ck/Ger/AR1559/2015	B	1a	neg	neg	N/A	N/A

(B) Environmental samples

	29 July 2015			Real-time RT-PCR			
		Farm	Stable	M PCR	H7	H7 HP	H7 LP
145	A/env/Ger/AR1526/2015	B	2b	33.83	neg	36.34	neg
146	AA/env/Ger/R1527/2015	B	2a	32.14	neg	26.83	31.86
147	A/env/Ger/AR1528/2015	B	2a	29.37	37.85	neg	35.54
148	A/env/Ger/AR1529/2015	B	2b	29.37	neg	neg	38.03
149	A/env/Ger/AR1530/2015	B	2b	37.87	37.56	35.04	neg
150	A/env/Ger/AR1531/2015	B	2b	33.4	neg	neg	36.29
151	A/env/Ger/AR1532/2015	B	2b	34.2	N/A	neg	N/A
152	A/env/Ger/AR1533/2015	B	2b	neg	N/A	N/A	neg

Threshold cycles for RT-qPCRs M and H7 PCR: ≤40; H7 LP and H7 HP PCR: ≤38

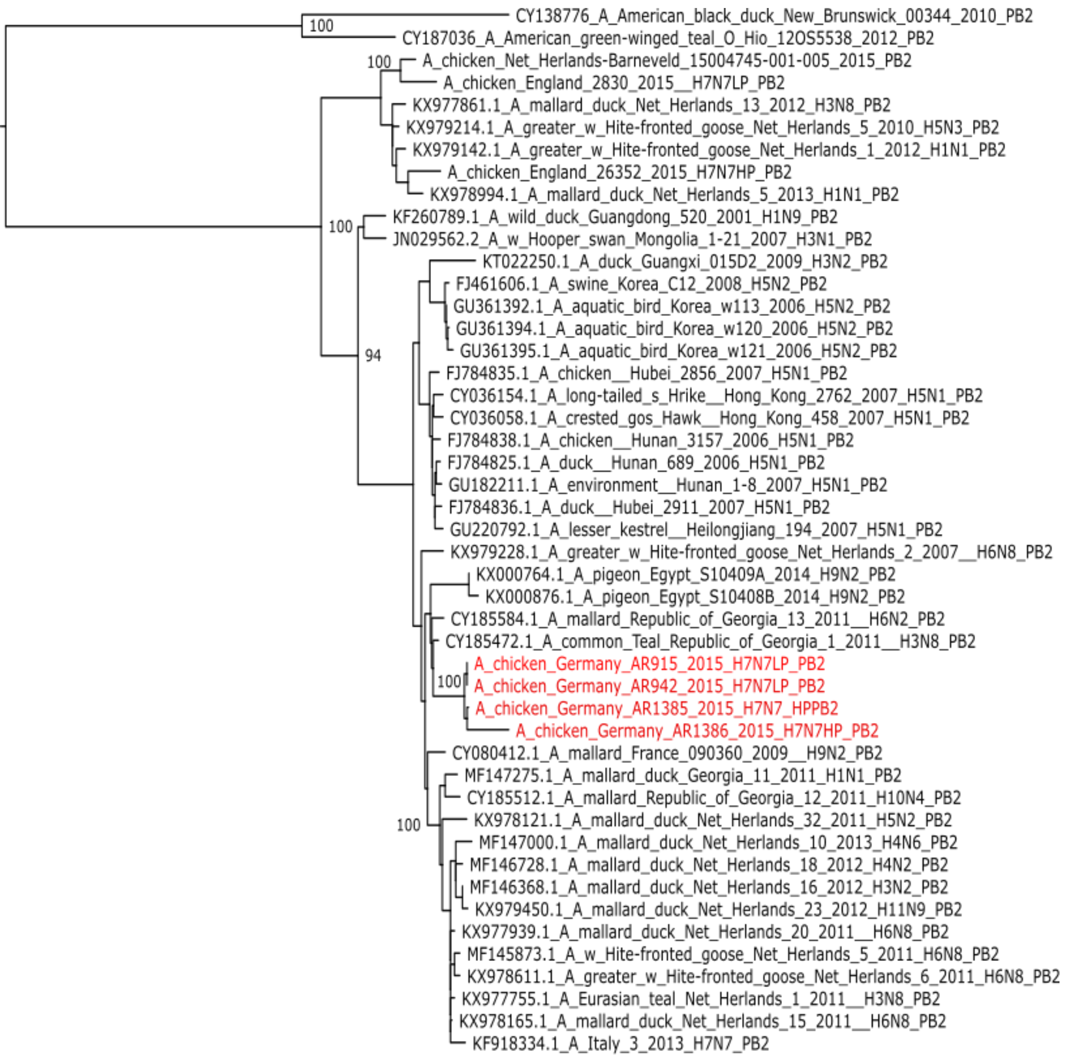
Neg, no positive signal detected (Cq > 40),

N/A, not applicable

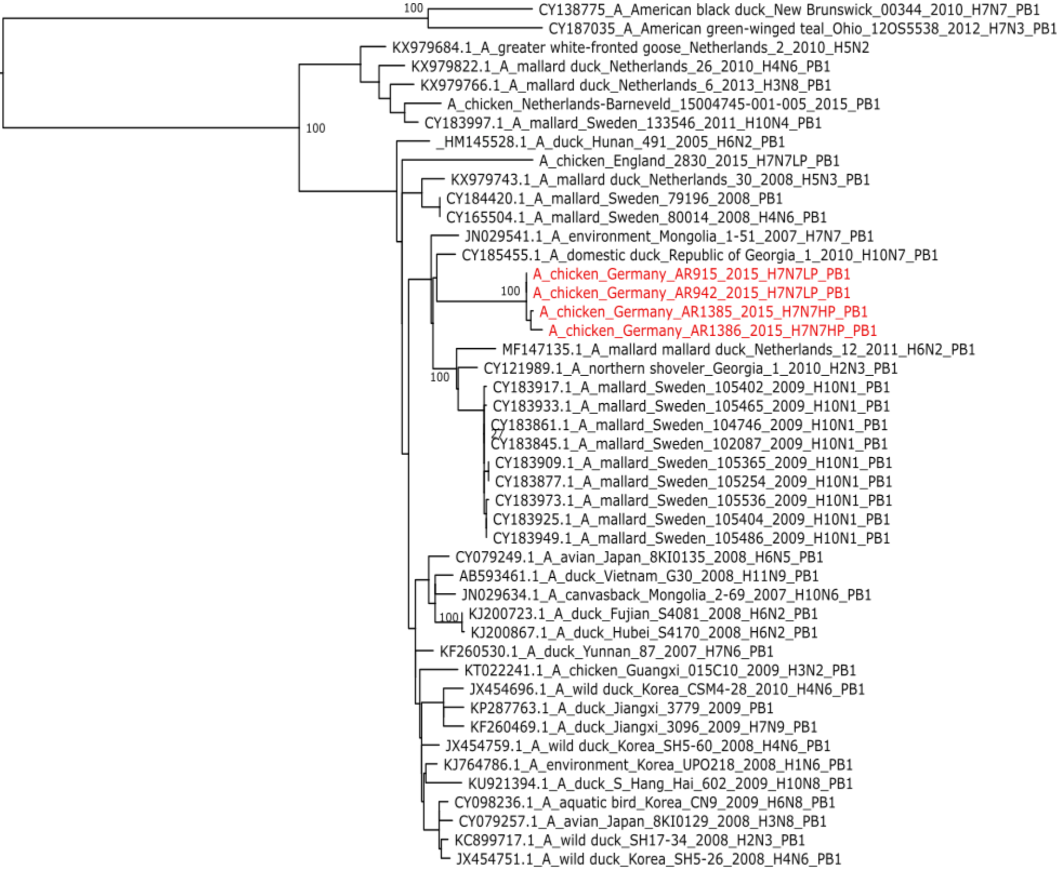
Green color: positive in at least one RT-qPCR; Yellow color: positive in virus isolation

Figures A-G: Phylogenetic analysis by maximum likelihood of genome segments of avian influenza viruses. Representative H7N7 viruses that were sequenced in this study are indicated in red.

(A) PB2



(B) PB1



(C) PA

100 CY138774_A_American_black_duck_New_Brunswick_00344_2010_PA
CY187034_A_American_green-winged_teal_Ohio_12OS5538_2012_PA
CY183577.1_A_mallard_Sweden_101165_2009_H8N4_PA
100 KX978459.1_A_mallard_duck_Netherlands_18_2010_H6N8_PA
KX979147.1_A_greater_white-fronted_goose_Netherlands_2_2011_H6N2_PA
CY166085.1_A_mallard_Sweden_124228_2010_H4N6_PA_
A_chicken_Netherlands-Barneveld_15004745-001-005_2015_PA
100 A_chicken_England_26352_2015_H7N7HP_PA
HQ014759.1_A_wild_duck_Korea_SH60_2004_H1N1_PA
EF597429.1_A_duck_Jiang_Xi_3345_2005_H5N2_PA
EF597405.1_A_duck_Jiang_Xi_1286_2005_H5N2_PA
CY109225.1_A_duck_Guangxi_1074_2006_H6N2_PA_
CY109233.1_A_duck_Guangxi_1157_2006_H6N2_PA
CY185422.1_A_mallard_Republic_of_Georgia_1_2010_H10N7_PA
CY185686.1_A_mallard_Republic_of_Georgia_2_2012_H2N3_PA
CY121988.1_A_northern_shoveler_Georgia_1_2010_H2N3__PA
CY185438.1_A_mallard_Republic_of_Georgia_3_2010_H7N3_PA
CY185406.1_A_common_coot_Republic_of_Georgia_1_2010_H6N2_PA
KF454778.1_A_wild_bird_Mongolia_2066_2011_H5N3_PA
MF146943.1_A_mallard_duck_Netherlands_22_2013_H2N3
100 MF145875.1_A_tufted_duck_Georgia_1_2012_H2N3_PA
100 CY185344.1_A_tufted_duck_Republic_of_Georgia_1_2012_H2N3_PA
JF789601.1_A_mallard_Czech_Republic_13438-29K_2010_H11N9_PA
MF146186.1_A_mallard_duck_Georgia_3_2012_H7N3_PA
MF145981.1_A_mallard_duck_Netherlands_2_2013_H3N6_PA
KX978941.1_A_greater_white-fronted_goose_Netherlands_1_2012_H1N1_PA
MF145734.1_A_mallard_duck_Netherlands_8_2014_H7N5_PA
AB807871.1_A_duck_Vietnam_LBM300_2012_H10N2_PA
KF874480.1_A_wild_waterfowl_Dongting_C2383_2012_H1N2_PA
KJ881018.1_A_duck_Guangxi_125D17_2012_H4N2_PA
KU161042.1_A_duck_Sichuan_S4202_2011_H4N2_PA
KP658042.1_A_duck_Yunan_V286_2012_H1N2_PA
MF147788.1_A_mallard_duck_Netherlands_18_2014_H1N1_PA
KX000723.1_A_pigeon_Egypt_S10409A_2014_H9N2_PA
KX014885.1_A_duck_France_150236_2015_H5N9_PA
A_chicken_Germany_AR915_2015_H7N7LP_PA
100 A_chicken_Germany_AR942_2015_H7N7LP_PA
A_chicken_Germany_AR1385_2015_H7N7HP_PA
A_chicken_Germany_AR1386_2015_H7N7HP_PA
KX978751.1_A_mallard_duck_Netherlands_24_2013_H4N6_PA
A_chicken_England_2830_2015_3_H7N7LP_PA
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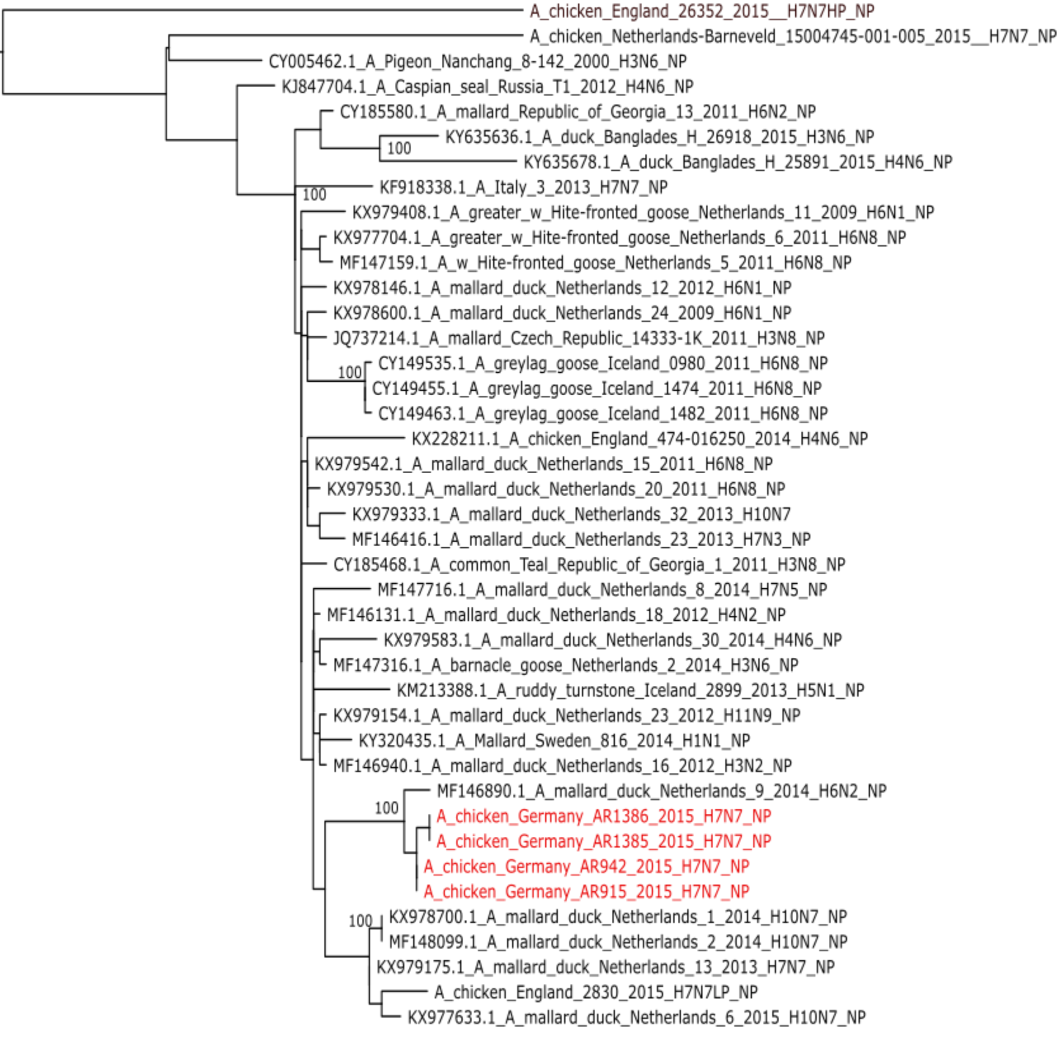

(E) NA

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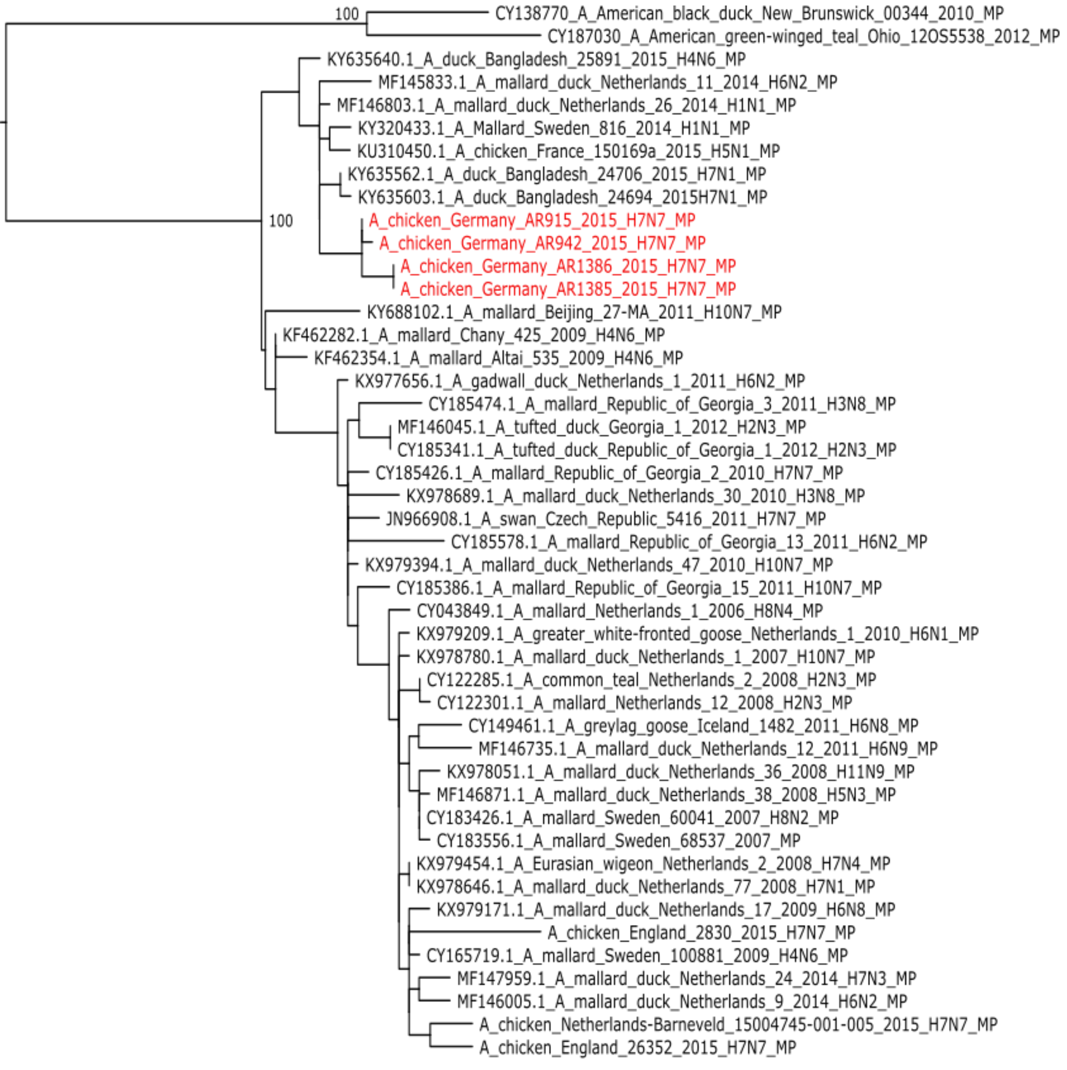
(F) MP



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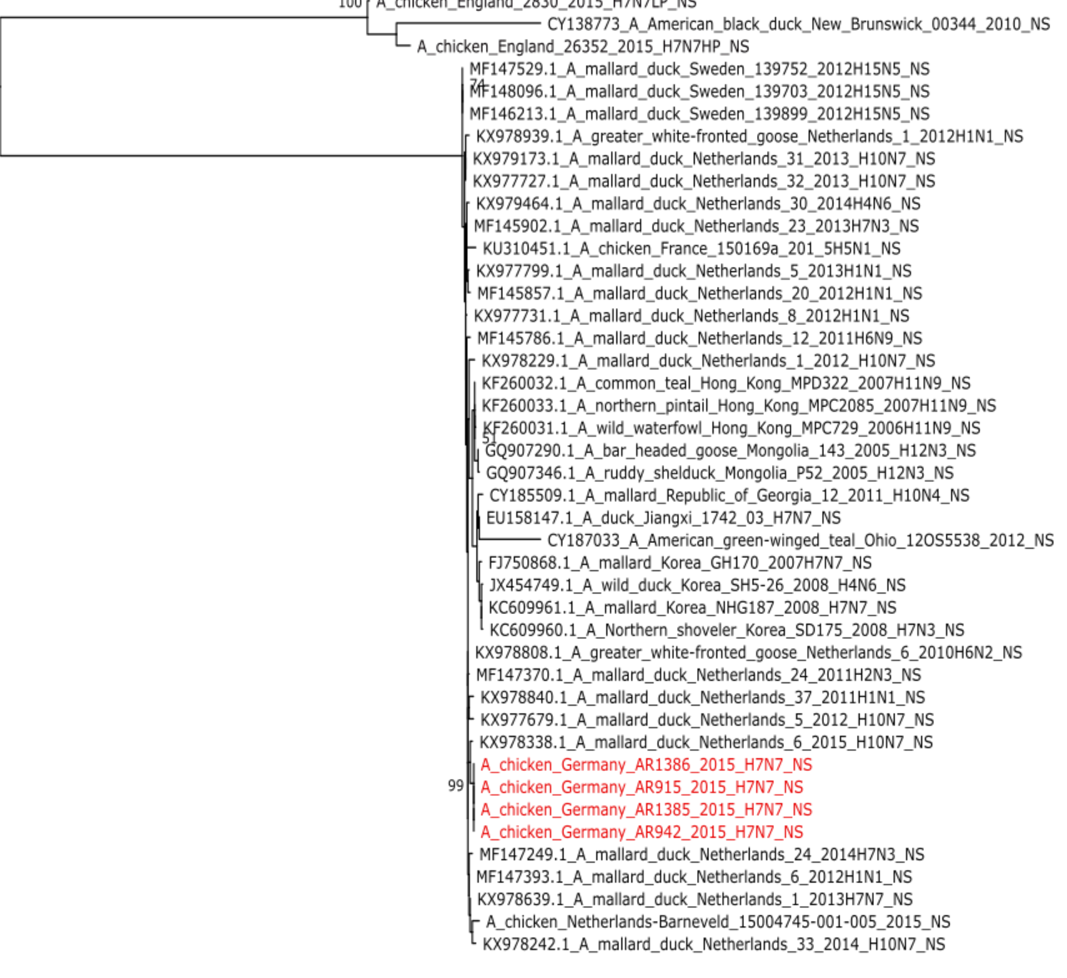

4.4 PUBLICATION IV

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

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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

Annika Graaf[1], Reiner Ulrich[2], Pavlo Maksimov[3], David Scheibner[4], Susanne Koethe[1], Elsayed M. Abdelwhab[4], Thomas C. Mettenleiter[4], Martin Beer[1] and Timm Harder[1]

Abstract

Highly pathogenic avian influenza virus (HPAIV) infection in poultry caused devastating mortality and economic losses. HPAIV of subtypes H5 and H7 emerge from precursor viruses of low pathogenicity (LP) by spontaneous mutation associated with a shift in the susceptibility of the endoproteolytic cleavage site of the viral hemagglutinin protein from trypsin- to furin-like proteases. A recently described natural pair of LP/HP H7N7 viruses derived from two spatio-temporally linked outbreaks in layer chickens was used to study how a minority of mutated HP virions after de novo generation in a single host might gain primacy. Co-infection experiments in embryonated eggs and in chickens were conducted to investigate amplification, spread and transmissionof HPAIV within a poultry population that experiences concurrent infection by an antigenically identical LP precursor virus. Simultaneous LPAIV co-infection (inoculum dose of 10^6 egg-infectious dose 50% endpoint (EID_{50})/0.5 mL) withincreasing titers of HPAIV from 10^1 to $10^{5.7}$ EID_{50}/0.5 mL) had a significant impeding impact on HP H7 replication, viral excretion kinetics, clinical signs and histopathological lesions (in vivo) and on embryo mortality (in ovo). LP/HP co-infected chickens required a hundredfold higher virus dose (HPAIV inoculum of 10^5 EID_{50}) compared to HPAIV mono-infection (HPAIV inoculum of 10^3 EID_{50}) to develop overt clinical signs, mortality and virus spread to uninfected sentinels. Escape and spread of HP phenotypes after de novo generation in an index host may therefore be highly precarious due to significant competition with co-circulating LP precursor virus.

Introduction

Avian influenza A viruses (AIV) are classified into subtypes based on antigenic properties of their hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins[1]. A further classification into phenotypes of low and high pathogenicity (LP/HP) refers to their virulence in chickens. In the subtypes H5 and H7, the HP phenotype correlates with presence of multiple basic amino acids at the HA endoproteolytic cleavage site (HACS) rendering it accessible to subtilisin-like proteases that are ubiquitous in avian host tissues[2–4].The vast majority of AIV circulating in their natural host reservoir of aquatic wild birds is identified as LP, encoding monobasic, i.e., trypsin-sensitive HACSs, and causing only minor, if any, clinical signs in avian hosts, including poultry[5]. LP viruses of the subtypes H5 and H7 have the ability, under natural conditions, to spontaneously mutate to the HP phenotype which is associated with conversion of the HACS from a trypsin-sensitive/monobasic to a subtilisin-sensitive/polybasic configuration[6]. The concept of HPAIV emergence from LP progenitors is supported by phylogenetic

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analyses of H5 and H7 AIV strains, which revealed the evolution of geospatially defined lineages within LPAIV, but not HPAIV phenotypes[7,8]. So far, only subtypes H5 and H7 AI viruses have been observed to acquire HP mutations under natural circumstances, and infection of poultry with these viruses are therefore considered to be notifiable regardless of their particular pathogenicity[9,10]. Control of notifiable LPAIV of subtypes H5 and H7 in poultry aims at preventing spontaneous mutation to and spread of HPAI variants associated with vast economic losses in poultry production. Some LPAIV such as the H7N9 strain in China merit control also because of their unique zoonotic propensity[11].

Repeated, but epidemiologically unrelated, emergence of HPAI H7 viruses based on de novo generation from distinct LPAIV precursors was at the basis of major outbreaks in Italy 1999–2000 (H7N1; ref.[12]) and 2013 (H7N7; ref.[13]), in the Netherlands in 2003 (H7N7; refs.[14,15]), in Canada in 2004 (H7N3; ref.[16]), in the United States of America in 2016 (H7N8; ref. [17]) and 2017 (H7N9; ref.[18]), in the UK in 200819,20 and Germany in 2015 (H7N7; ref.[19]). H7 HPAIV detection in isolated, sporadic outbreaks was reported from the UK in 2015 (H7N7; ref.[20]), from Canada in 2007 (H7N3; ref.[21]) and from Spain in 2009/2010 (H7N7; ref.[22]).

Virological identification of a "matching pair" of an LPAIV progenitor and its HPAIV descendant in the field is very rare[23–25]. A recent example of such a virus pair was reported from Germany where transmission of a precursor H7N7 LPAIV from chicken layer farm A to neighboring layer farm B and mutation to the HP phenotype on farm B was confirmed[19].Determining the drivers of emergence of HPAIV is crucial for a better understanding why and when certain LP strains pose a risk of becoming HP. There is insufficient knowledge why, in nature, the HP phenotype emerges only in H5 and H7 subtypes and how the two AIV pathotypes interact when simultaneously infecting poultry. We conducted in vivo and in ovo co-infection experiments using a naturally occurring matching LP/HP H7N7 virus pair to further understand the processes of the initial emergence and escape of HPAIV. Our experiments attempted to mimic the situation of the de novo emergence and spread of an HPAIV infection in a chicken population in which LPAIV is circulating.

Results

In order to mimic the status nascendi,when an HP phenotype variant emerges by spontaneous mutation from an LP precursor virus in an avian host that is infected by the LP phenotype, groups of ten 6-week-old specific pathogen free (SPF) chickens or five SPF embryonated chicken eggs (ECEs) of either 10-days or 14-days of incubation, respectively, were co-inoculated with an HP/LP virus mixture containing increasing titers of HPAIV H7N7 AR1385from 10^1 to $10^{5.7}$ EID_{50}/animal (groups C1-C5.7, C = co-infected, number = $\log_{10}$ virus titer) in a constant background titer (10^6 EID_{50} /animal) of the LPAIV H7N7 AR915 precursor. As a comparison, similar inoculation of chickens and eggs were carried out with corresponding HPAIV doses without concomitantLPAIV infection (HP mono (M)-infected groups M1-M6). In addition, we also included a group which solely received the LPAIV precursor (group B).

In vivo experiment

Clinical score and survival rate of chickens reveal interference of LP with low titer HP H7N7 infection

Neither morbidity (quantitative measure: clinical score; Fig. 1a) nor mortality (quantitative measure: survival probability, Fig. 1b) was observed in any bird of group B inoculated with 10^6 EID_{50} of LPAIV H7N7 AR915 as a mono-infection (Fig. 1a, b: B vs. M6, blacklines). Dose-dependent morbidity and mortality was evident in groups that received an inoculum of HPAIV H7N7 AR1385, the HP successor of LPAIV AR915, as a mono-infection (groups M1-M6, Fig. 1a, b, turquoise lines). Animals of group M6 which received the highest HPAIV dose (10^6 EID_{50}/0.5 mL) presented with severe lethargy, anorexia and neurological signs leading to a moribund status as defined in humane termination criteria (see Materials and Methods) of all inoculated birds within 3 to 4 days post infection (dpi) (mean clinical score = 2.1). As expected, highly significant differences between the control groups LP B and HP M6 in survival rates and clinical scores of inoculated animals were observed (each $p < 0.001$) which confirms the validity of the inoculation and scoring system (Fig. 1a, b, B vs. M6, supplemental Table 1a, b).

Average clinical scores of other HPAIV challenged groups decreased with decreasing inoculum titers from 2.0 (in group M5.7), 1.9 (in group M5) and 1.6 (in group M4) to 1.4 (group M3). A delay in mortality but no difference in the terminal severity of clinical signs was observed for the groups M3 and M4 compared to groups M5, M5.7, and M6 (Fig. 1a, b, turquoise lines). However, mortality did not reach 100% in groups M3 and M4 and, thus, survival probability in groups M3 and M4 was significantly higher (each, $p < 0.001$) than in M5-M6. In group M1, in contrast, neither clinical signs nor mortality were observed. In conclusion, as a mono-infection, at least 10^3 EID50 of the HPAIV AR1385 inoculum per animal were required to induce robust infection, clinical signs and mortality.

Chicken groups C1-C5.7 (Fig. 1a, b, orange lines) had been co-infected with an inoculum containing a constant amount of LP AR915 (10^6 EID_{50}/animal) and varying titers of HP AR1385 matching those of the mono-infected groups. Compared to the HPAIV mono-infected

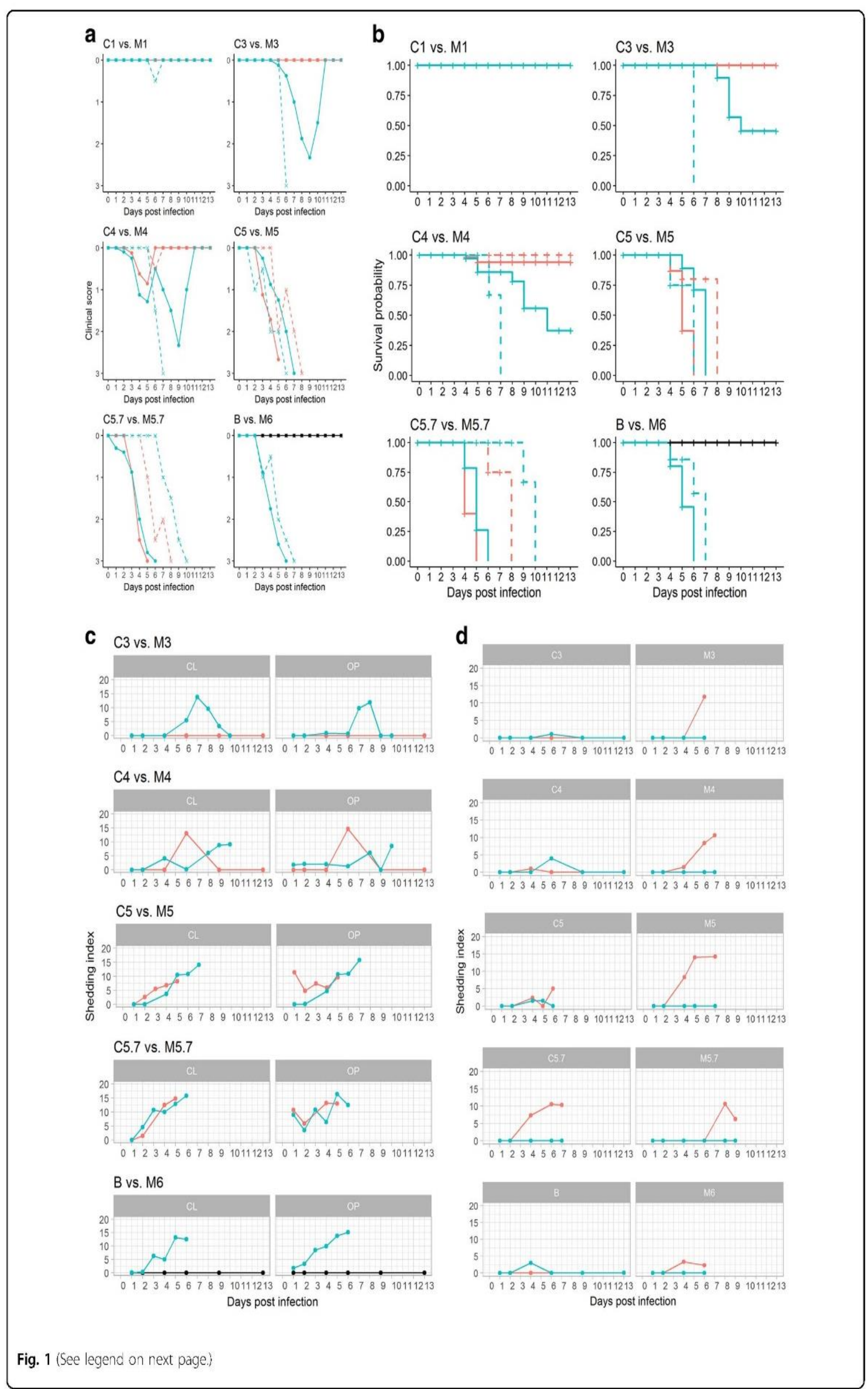

Fig. 1 (See legend on next page.)

(see figure on previous page)
Fig. 1 a-b: Clinical scores (**a**) and survival probability (**b**) of chickens after co-infection with LP/HPAIV H7N7 (groups C1-C5.7, orange lines) compared with titer-matched HP H7N7 mono-infected groups (M1-M5.7, turquoise lines), and comparison between titer-matched LP (B, black line) and HP H7N7 (M6, turquoise line) mono-infected groups. Continuous lines represent inoculated animals; dashed lines depict sentinels. **c–d**: Oropharyngeal (OP) and cloacal (L) shedding of HPAIV H7N7-specific RNA by infected (**c**), and sentinel (**d**) chickens after co-infection with LP/HPAIV H7N7 (groups C1-C5.7, orange lines) compared with titer-matched HP H7N7 mono-infected groups (M1-M5.7, turquoise lines), and comparison between titer-matched LP (B, black line) and HP H7N7 (M6, turquoise line) mono-infected groups. A pathotype-specific RT-qPCR was used to generate Cq values (Graaf, 2017). The shedding index (Y-axes) was calculated by computing the mean average of Cq values of all animals sampled at the indicated dpi in a specific group and subtracting this value from 38, the threshold of detection of the HP H7 RT-qPCR. Thus, if all animals were negative for HP H7 RNA, the group scored with a value of zero

groupsthey showed an attenuated course of disease: HPAIV added to the LPAIV inoculum at doses of 10^1 EID_{50} (group C1) and also 10^3 EID_{50} (group C3) did not induce any signs of disease or mortality. Thus, there was a significant difference between group C3 (no morbidity/mortality) vs. M3 (9/10 birds died) regarding survival probability and clinical scores (each $p < 0.001$). An HPAIV co-infection dose of 10^4 EID_{50} (group C4) infrequently caused very discrete clinical signs in chickens. Spontaneous mortality was induced in two inoculated birds of this group at 4 and 5 dpi that did not show any preceding clinical signs (C4, meanclinical score = 0.6; Fig. 1a, b, orange lines); this reveals a highly significant difference to group M4 (survival probability $p = 0.002$; clinical score $p = 0.002$) (supplementalTable 1a, b).

High morbidity and mortality rates, evidently indistinguishable (survival probability and clinical scores $p > 0.2$) from HPAIV mono-infection, were induced by mixed inocula containing 10^5 and $10^{5.7}$ EID_{50} HPAIV in groups C5 and C5.7 (Fig. 1a, b; supplemental Table 1a, b), respectively.

The only significant difference emerged within the survival rates between groups C5 vs. M5 in inoculated animals ($p << 0.001$). In conclusion, when compared to HPAIV mono-infections, an attenuated course of disease was evident when less than 10^5 EID_{50} of the HPAIV were mixed with an inoculum of 10^6 EID_{50} of the LPAIV.

HP H7N7 virus shedding is impeded by LP co-infection

Oropharyngeal (OP) and cloacal (CL) viral shedding patterns were examined by generic AIV-M-specific and pathotype-specific H7 LP and H7 HP RT-qPCRs (Hoffmann, 2001; Graaf et al., 2017). The total number of positive swabs, the viral loads and the duration of virus shedding varied among the groups (Fig. 1c and supplemental Table 2). The threshold distinguishing positive and negative samples was set at Cq = 40 for the M- and at 38 for both, the H7 LP and HP specific PCRs. The latter PCRs were found to be slightly less sensitive leading to more M-positive versus H7-positive samples (supplemental Table 2). No H7 LPAIV-specific RNA was detected in any of the HPAIV mono-infected groups M1-M6, and likewise, no H7 HPAIV-specific RNA was detected in the LPAIV mono-infected group B, confirming the purity of the virus preparations and effective isolation of animal units.

All virus-inoculated chickens with the exception of group M1 and a single inoculated chicken in group M3 excreted AIV RNA in OP and/or CL swabs (supplemental Table 2). Chickens of the LPAIV mono-infected group B shed virus orally and cloacally starting from 1 and 2 dpi, respectively, and virus excretion in this group peaked at 4 dpi; even at 13 dpi, minor amounts of viral RNA were excreted by three out of ten chickens of this group (supplemental Table. 2). Virus excretion kineticsfor the HPAIV mono-infected chickens of group M6 (Fig. 1c, b vs. M6) showed a steady increase of virus shedding until death of the animals within 6 days. In co-infected groups, shedding of both LP-specific and HP-specific H7 RNA was evident, with the exception of group M1 where neither LP nor HP shedding was detected; with increasing amounts of HPAIV in the inoculation mixture, less LP H7-specific RNA was excreted (supplemental Table. 2).

In groups C1 and C3 no HP shedding was observed (C1 not shown in Fig. 1c, supplemental Table 2). Groups C5 and C5.7 did not shed statistically significant lower amounts of HPAIV RNA as compared to the HPAIV mono-infected groups M5 and M5.7 (Fig. 1c, supplemental Table 3b). Group C4 showed a singular HP excretion pattern with HPAIV RNA shedding receding to undetectable levels after a peak at 6 dpi; obviously co-infected chickens in this group cleared the HPAIV infection within the observation period. This is corroborated by the clinical picture where 6/8 inoculated birds survived.

HP H7N7 virus transmission from LP-co-infected donors to sentinel chickens requires higher HP inoculum doses

Virus transmission kinetics were assessed by co-housing four sentinel chickens with each of the groups at 1 dpi. Two sentinels each were sacrificed for immunohistochemistry (IHC) at 2 dpi. Effective transmissionwas evaluated by the development of morbidity/mortality (Fig. 1a, b dashed lines), presence of AIV RNA in OP and

CL swab samples (Fig. 1d), and by seroconversion (supplemental Fig. 1) of the two remaining sentinels.

All contact chickens of HPAIV groups M and C, except those of groups C1-C4 and M1, excreted HPAIV (Fig. 1d and supplemental Table. 2), developed clinical signs and succumbed to the infection (Fig. 1a, b). Transmission of LPAIV as judged by virus excretion of sentinels was evidentby LP H7 pathotype-specific RT-qPCR in groups B, and C1-C5. No LPAIV excretion could be demonstrated in sentinels of group C5.7 (Fig. 1d, supplemental Table 2).

In summary, highly significant differences between the control groups B and M6 in survival and clinical score of sentinel animals were observed (each $p < 0.001$) (Fig. 1a, b). Furthermore, groups C3 vs. M3 and C4 vs. M4 showed a significant difference in survival ($p < 0.001$), while clinical scores did not differ significantly ($p > 0.05$). No statistical differences were observed in the remaining groupsC1 vs. M1, C5 vs.M5, and C5.7 vs. M5.7.

Results of the AIV NP antibody-specific seroconversion are shown in supplemental Fig. 3. All chickens found to be infected by molecular means and surviving until the end of the observation period (13 dpi) seroconverted.

Lack of macroscopic and histopathological findings in LP H7N7 infected chickens but characteristic lesions in HP H7N7 inoculated chickens at day 2 post infection

Necropsy of four chickens of each group (two inoculated and two sentinel birds) sacrificed at 2 dpi revealed no conspicuous pathological signs for AIV infection in either inoculated or contact chickens. This includes birds of all HPAIV mono-infected groups. Chickens inoculated with LPAIV and sacrificed at 2 dpi revealed no viral antigen-positive cells and no obvious histopathological alterations (supplemental Fig. 3).

In HPAIV infected chickens, in contrast, the most abundant histopathological finding at 2 dpi was mild to moderate, focal to multifocal, acute necrotizing rhinitis with epithelial degeneration and necrosis (groups M5-M6 and C4-C5.7; Fig. 2a; supplemental Fig. 2). Less frequently present was mild oligofocal, acute degeneration and necrosis of individual caecal crypt epithelia (Fig. 2c; supplemental Fig. 2) and mild oligo-focal to multifocal, acute, necrotizing polioencephalitis (Fig. 2e; supplemental Fig. 2a), respectively. Further mild to moderate lesions in other tissues are shown in Fig. 2g; supplemental Fig. 2b and 2d.

These results were corroborated by IHC revealing most abundant, oligofocal to coalescing influenza A matrixprotein-positive epithelial cells within the nasal cavities (Fig. 2b; supplemental Fig. 2c), caecal crypt epithelia (Fig. 2d; supplemental Fig. 2c), and the brains of chickens with polioencephalitis, respectively (Fig. 2f; supplemental Fig. 2c). Oligofocal, immunoreactive round cells interpreted as macrophages were present within the spleens of one to two chickens of groups M5.7, M6, and C5.7 (Fig. 2h; supplemental Fig. 2c). Other organs with scant focal immunoreactive parenchyma in individual chickens of groups M5-M6 were heart, lungs, and liver (supplemental Fig. 3C).

In ovo experiment

ECEs at both 10 and 14 days of age were inoculated with either H7N7 LP AR915 or HP H7N7 AR1385 or mixtures thereof in 0.2 mL per egg as shown in Table 1b.

LP H7N7 interferes with HP infection of embryonatedchicken eggs

Mean death time (MDT) values within an observation period of 4 days and viral load in amnio-allantoic fluids (AAFs) were used to compare mono- and co-infection groups. Significantly increased mortality and shortened MDT in groups M6 versus B, representing mono-infections with HP AR1385 and LP AR915 (inoculum each of 10^6 EID_{50}), was evident ($p < 0.0001$; quantitative measure: survival probability, Fig. 3a, B vs. M6, supplemental Table 4a,b). No significant ($p > 0.5$) difference was observed when comparing 10-day old and 14-day old eggs for each of these groups. Examination of virus loads in AAFs by generic AIV-M and pathotype-specific H7 LP- and H7 HP RT-qPCRs revealed pure LP and HP infections in the mono-infected groups B and M6, respectively (Fig. 3b, supplemental Table 5).

Independent of the virus dose at inoculation, the MDT of HP mono-infected ECEs was always significantly shorter, i.e., they died earlier, compared to LP/HP co-infected eggs (Fig. 3a, supplemental Table 4b). In addition, HP virus yield in all co-infected groups was strikingly lower compared to mono-infectionsbut increased with increasing HP inoculum titers,whereas no significant differences ($p > 0.05$) in LP virus load of co-infected groups was evident; interference of HP virus by LP co-infection was particularly dominant in 10-day old ECEs (Fig. 3b, supplemental Table 5). The only exceptions are groups C1 and M1 where no embryos died in M1, although all M1 eggs became infected (Fig. 3a, supplemental Table 5). In contrast, the death of all 5 (10-day old ECEs) and 3 of 5 ECEs (14-day old) in C1 must have been attributable mainly to LP replication as confirmed by pathotype-specific RT-qPCR (Fig. 3b, supplemental Table 5). ECEs inoculated at 10 days of incubation had a significantly shorter MDT compared to 14-day old ones in the co-infected but not in the mono-infected groups (Fig. 3a, supplemental Table 4b). However, this correlation was blurred by HPAIV mono-infections, and HP virus yield in AAFs did not vary with the inoculum dose and age of the ECE (Fig. 3b, supplemental Table 5).

Endotheliotropic-vascular systemic virus spread is characteristic for HP H7N7 in ECEs

The trilamellar structure of the chorioallantoic membrane (CAM; inner layer = allantoic epithelium,

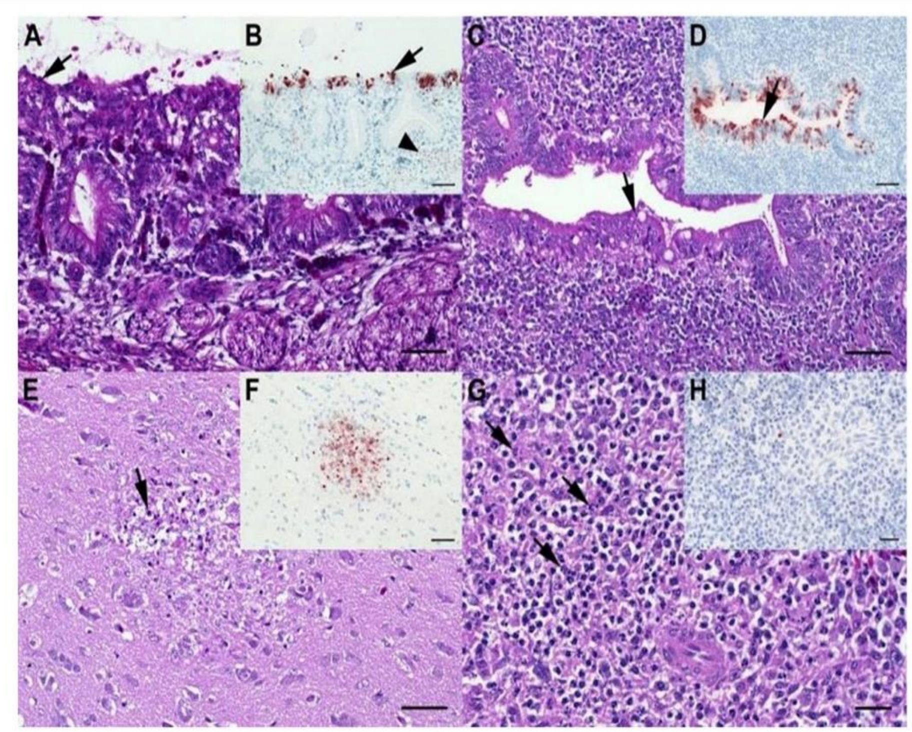

Fig. 2 Histopathological findings in chickens (scheduled euthanasia at dpi 2) characteristic for HPAI. a Chicken, P17–882, group C5.7, respiratory mucosa. Moderate, multifocal, acute, necrotizing rhinitis with epithelial degeneration characterized by attenuation, loss of cilia (arrow), and sloughing. **b** Chicken P17–882, group C5.7, respiratory mucosa with coalescing foci of influenza A matrixprotein-immunoreactive ("antigen-positive") morphologically intact and degenerated epithelial cells (arrow). Notably, there are scant immunoreactive granules in some of the submucosal nerves (arrowhead), suggestive of axonal spread. **c** Chicken, P17–903, group M5.7, cecum. Mild, oligofocal, acute, crypt epithelial degeneration with nuclear pyknosis (arrow), necrosis and sloughing. **d** Chicken, P17–903, group M5.7, cecum. Antigen-positive morphologically intact and degenerated crypt epithelia (arrow). **e** Chicken, P17–903, group M5.7, brain. Mild, oligofocal, acute, necrotizing polioencephalitis characterized by neuroglial cytoplasmic hypereosinophilia, nuclear pyknosis, karyorrhexis and loss (arrow), as well as an associated *status spongiosus* interpreted as inflammatory edema. **f** Chicken, P17–903, group M5.7, brain. Antigen-positive neuroglial cells within necrotizing lesion. **g** Chicken, P17–903, group M5.7, spleen. The periarteriolar lymphoid sheaths and follicles display moderate, coalescing apoptotic lymphocytes characterized by cytoplasmic hypereosinophilic shrinkage and nuclear karyorrhexis (arrows). **h** Chicken, P17–903, group M5.7, spleen. Rare, individual antigen-positive round cells can be detected in the white and red pulp. **a**, **c**, **e**, **g**: Hematoxylin eosin. **b**, **d**, **f**, **h**: Influenza A virus-matrixproteinIHC. **a**–**f**: bar = 50 µm. **g**, **h**: bar = 20 µm

intermediate mesenchymal layer and outer layer = chorionic epithelium) was clearly discernable in all investigated eggs/embryos[26]. Overall distribution patterns of virus antigen in the CAM and embryonal organs displayed a comparable pattern in 10-day old and 14-day old eggs (Fig. 4), and were attributable to the viral pathotype: Infection with LPAIV (embryos of group B) was mostly confined to the allantoic epithelium of the CAM (Fig. 4). In contrast, infection with HPAIV affected both the allantoic and chorionic epithelial layers, as well as endothelial cells within blood vessels of the mesenchymal layer of the CAM (Fig. 4, supplemental Figs. 4C and 4E). Furthermore, endotheliotropic systemic virus dissemination via the vascular system in embryos co-infected with HPAIV (groups C1-C5.7) was prominent with viral antigen present in endothelial and parenchymal cells of various internal organs (CNS, lung, liver, gizzard, and various parts of the gastrointestinal tract, and in some cases in the kidneys) (supplemental Fig. 3, supplemental Fig. 4C-F).

Examination of embryonic parenchymal organs (brain, liver, heart) by H7 pathotype-specific RT-qPCR revealed H7 LP RNA in a minority of tissue samples, mainly of 10-day old ECEs of some co-infection groups (C1-C5.7; supplemental Table 6a, red-colored values). Contamination from AAF during preparation cannot be fully excluded although no LP H7 RNA was detected in embryonic tissues of group B (LP H7 mono-infection). Presence of HPAIV RNA wasconfirmed in heart, brain and, liver tissues of both, 10-day old and 14-day old ECEs (supplemental Table 6a) but not in embryos of group B.

Discussion

A shift of the susceptibility of the HACS from trypsin- to furin-like endoproteases essentially governs the

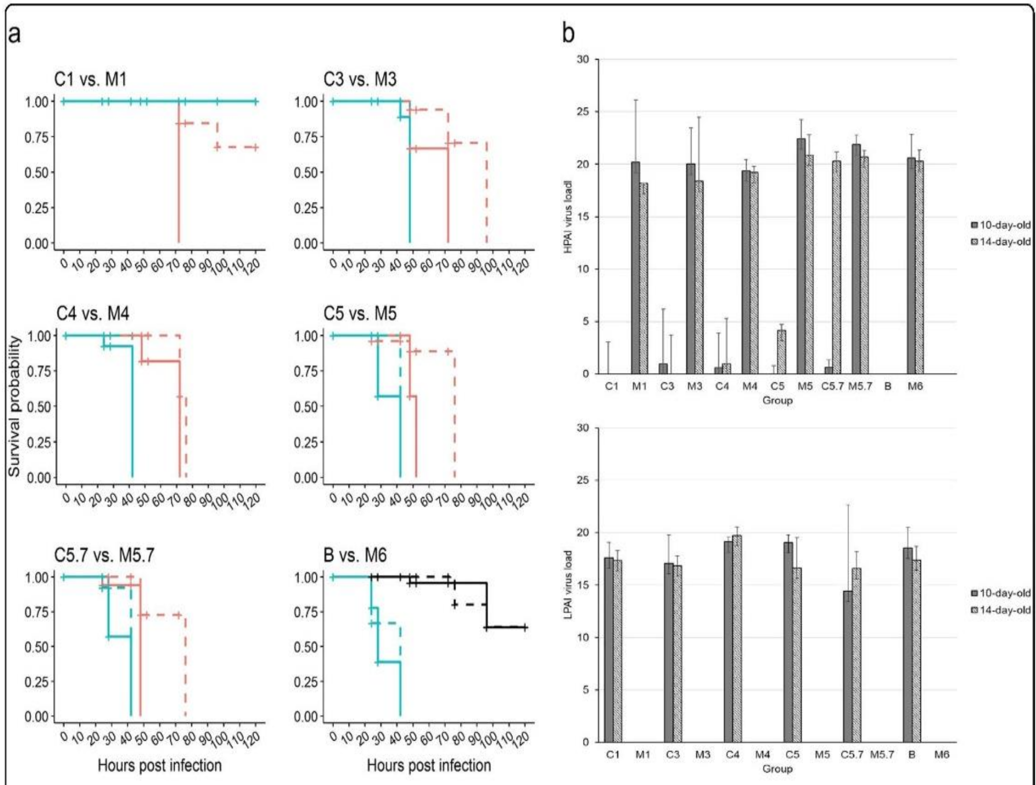

Fig. 3 Results of the in ovo experiment. a. Survival probability of embryonated chicken eggs (ECEs) inoculated at 10 or 14 days of incubation with LP (group B, black lines in lower right panel "B vs. M6") or HPAIV H7N7 (groups M1-M6, turquoise lines) or with LP/HP mixtures according to Table 1b (groups C1-C5.7, orange lines). Solid lines represent ECEs 10-day old and dashed lines 14-day old at inoculation. **b**. HP (upper panel) and LPAI (lower panel) H7N7 virus loads in amnio-allantoic fluids of ECEs aged 10-and 14-days at inoculation determined by H7 pathotype-specific RT-qPCRs (mean values representing "virus load" were calculated by substracting the measured Cq value from the threshold of detection at Cq = 38; individual values are presented in supplemental Table 3a)

emergence of an HP variant from its LP precursor[13,19,20,22,27,28]. Insertion of multiple basic amino acids[29–31] but also intersegmental recombination and insertion of amino acids that fostered a bulging of the HACS[25,32,33] mediated this switch in pathogenicity. Factors remained unknown that govern the likelihood of such spontaneous LP-to-HP mutations and skew the initial amplification and spread of the HP variant to ensure primacy over its LP precursor in the index bird. As we deeply studied the mutations of the applied LP/HPAIV matching pair in a former study, none of these mutations have been assigned to any specific functions, although the mutation N92D in NS-1 affected a position that has been associated with interferon escape[19]. We therefore performed co-infection experiments of LP and HP H7 viruses in chickens in vivo and in ovo to mimic the initial moments of amplification and spread following the de novo generation of HP variants. Subsequently, the HP variant must be transmitted within a chicken population, where a concurrent infection of the antigenically identical LP precursor is likely on-going as was the case in the layer flock from which our HP H7 variant originated[19].

Our results demonstrated interference between LP and HP viruses that turned out to be highly unfavorable for the HP phenotype to gain primacy at LP/HP ratios exceeding 100:1. The factors responsible for interference have not been elucidated here but it seems likely to suspect that innate immune mechanisms such as interferon activity are involved[34].We speculate that a switch to the HP phenotype has to occur very early after LPAIV infection to avoid interference by high loads of replicating LPAIV. In these cases, as mimicked by our co-infected groups C5 and C5.7, the HP variant prevailed, and substantial amounts of HP sufficient to infect sentinel animals were excreted. In contrast, in other co-infected groups receiving an inoculum with an unfavorable LP/HP ratio, such as C3 and C4, hampered HP virus excretion resulted, and transmission to sentinels failed. In these cases, HP mutants seemed to be unable to spread from the index animalwhile LP virus was excreted via oropharyngeal secretions and feces (groups C3 and C4).

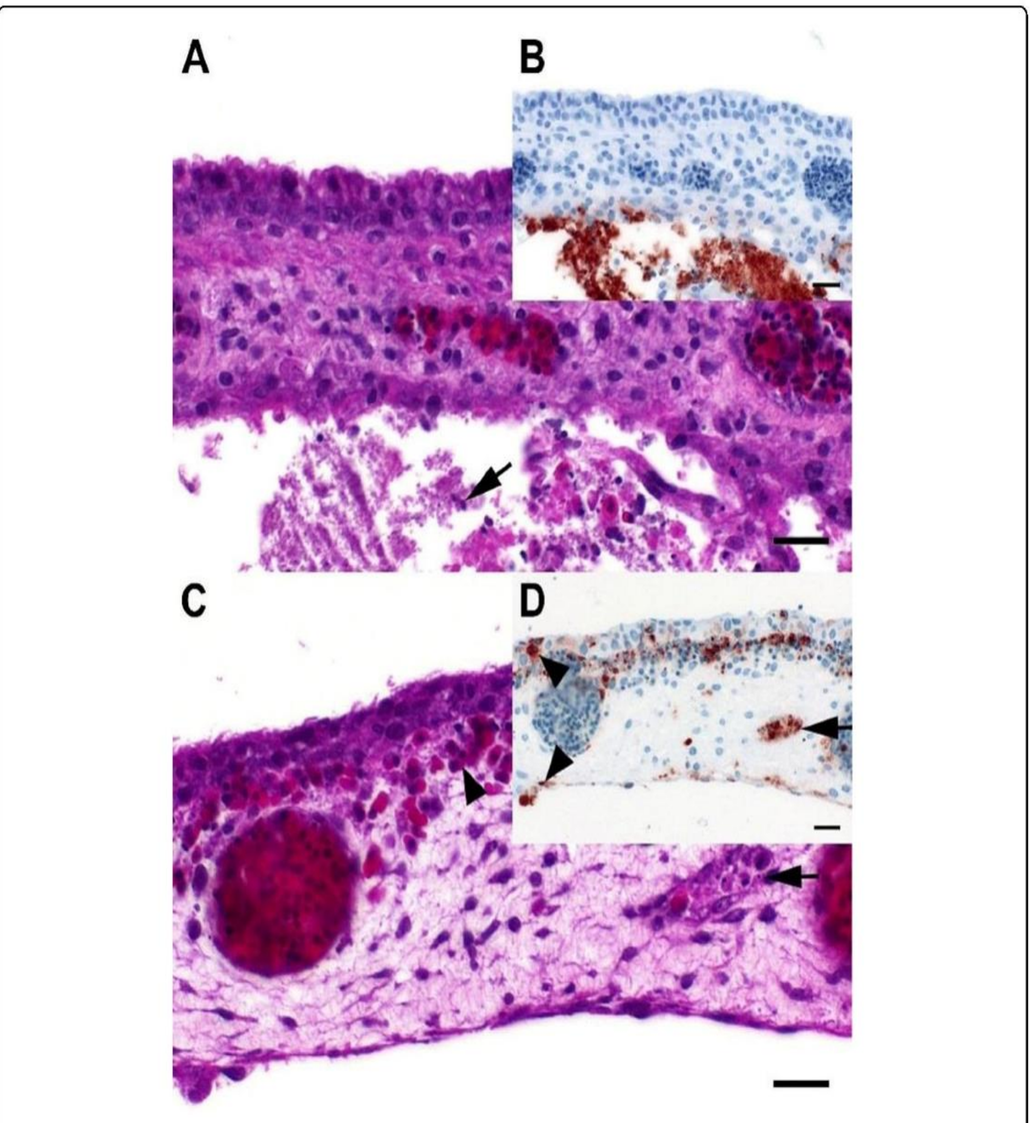

Fig. 4 Histopathological findings in embryonated chicken eggs characteristic for LP and HP H7N7influenza A viruses exhibitingdistinct tropism in the chorioallantoic membrane. **a** Chicken, 14-day old embryo, P18–103, group B, 4 dpi, chorioallantoic membrane. There is necrosis and loss of the allantoic membrane and accumulation of cellular debris (arrow) and fibrin in the allantoic sac. **b** Chicken, 14-day old embryo, P18–103, group B, 4dpi, chorioallantoic membrane. Influenza A matrixprotein-immunoreactive ("antigen-positive") cells and cellular debris accumulations restricted to the allantoic side of the chorioallantoic membrane. **c** Chicken, 12-day old embryo, P18–46, group C5.7, 4dpi, chorioallantoic membrane. Moderate, multifocal, acute, necrotizing vasculitis (arrow) with edema and extravascular accumulation of erythrocytes (arrowhead) interpreted as hemorrhage. **d** Chicken, 12-day old embryo, P18–46, group C5.7, 4dpi, chorioallantoic membrane. Influenza A-immunoreactivity is abundant in vascular endothelia (arrow) and blood-borne leukocytes and to a lesser degree epithelial cells (arrowheads) of both the chorionand the allantois (bottom). **a**, **c**: Hematoxylin eosin. **b**, **d**: Influenza A virus-matrixprotein IHC. **a**–**d**: bar = 20 µm

Escape of HP would depend on the death of the bird and exposure of susceptible hosts to systemically infected tissues. Such can be envisioned, e.g., by picking on decomposing carcasses[35], contact with infected feather cones[36–38], eggs[39,40] or on mechanical transmission by hematophagous ectoparasites such as *Dermanyssus spp*[41].

At the population level, transmission of HP mutants after escape from index birds depends on the presence of susceptible hosts. As former studies already showed, LPAIV precursor-specific immunity effectively reduced susceptibility to homologous HP infection and grossly decreasedHP transmission efficacy[42–44] resembling immunization with a perfectly matching modified live virus vaccine. At flock level, the emerging HP variant would benefit from (i) a low prevalence of LP-specific adaptive immunity to avoid specific immune interference,

and (ii) a low incidence of active LP precursor virus infection to prevent direct competition. Populations showing high seroconversion rates should be unlikely to propagate HP and may pose a low risk from an animal-disease-control perspective.

The in ovo experiment mirrored the inhibitory effects of an LP infection on the replication kinetics of co-inoculated HPAIV. We decided to compare 10-day old and 14-day old embryos due to developmental features in the embryo (e.g., new physical and functional barriers like the trilamellar structure of the CAM, access to tryptic proteases, etc.), that in turn may modify the distribution of AIV in embryonic tissues[45,46]. Characteristic differences regarding sites of replication of LP and HPAIVs in ECEs[47–50] were confirmed for the current natural LP/HP pair of subtype H7N7. In particular, IHC analyses of the trilamellar CAM allowed an easy and clear-cut distinction between LP (infection of allantoic epithelium only) and HPAIV (cells of all three lamellae affected with characteristic endotheliotropism and vasculitis). Rarely, in embryos of group B (mono-infected by LP), immune-labeled cells were also found in the surface epithelia of the skin, nasal cavity, respiratory, and upper alimentary upper alimentary tract including the stomach; this pattern is suggestive for a canalicular spread of virus provided it had reached the amniotic cavity. Although inoculation was targeted to the allantoic sac, accidental lesion of the amnion cannot be excluded[50,51]. We confirmed previous results suggesting an increased resistance of 14-day old versus 10-day old ECEs to AIV infection[29,52,53].

Considering all impediments that hamper HP variants to gain primacy over its LP precursor, the de novo emergence of HP viruses is likely a very rare event. Nevertheless, several de novo HP outbreakshave been detected during the last decade[8,17–19]. It is challenging to speculate that LP-to-HP mutation events in vivo actually might occur more frequently in LP-infected poultry flocksthan anticipated. Often, however, such conversion events might escape detection because HP virus remained trapped in the index birds or failed to spread in the population. Experimental LP-to-HP conversion of H7 virusesby serial passaging of LPAIVs has been successful[24,54]. Although HP phenotypes were generated eventually in several[29,55,56] but not all attempts, a high number of passages was usually required. Yet, the true conversion rate of LP-to-HP may be underestimated.

The presented clinical, pathological and virological data, obtained by in ovo and in vivo co-infection experiments using a natural pair of LP and HP H7N7 viruses, revealed an intricate interference between the two phenotypes. HP variants generated by de novo mutation need to overcome a series of obstacles both in the index bird and in the index population to gain primacy. The in ovo model showed potential to determine, by IHC, tissue tropism and pathogenicity of AI viruses. Exploring different routes of inoculation (e.g., allantoic versus amniotic versus vascular) in 14-day old ECEs and subsequent deep sequencing of selected embryo tissues may also be appropriate to select pathogenicity variantsgenerated de novo or propagated from a minority population in the quasispecies of an isolate.

Materials and methods

Ethics statement

All experiments were carried out in biosafety level-3 (BSL-3) laboratory and animal facilities at the Friedrich-Loeffler-Institute (FLI, Germany) with permission of the FLI biorisk committee in accordance with a protocol legally approved by the Ethics Commission of the Ministry of Agriculture and the Environment of the State of Mecklenburg-Vorpommern, Germany (LALLF MV 7221.3–1.1–039/17).

Virus origin and propagation

Viruses originated from two epidemiologically linked outbreaks in chicken layer farms in Germany in summer 2015. The two reference viruses constitute a natural pair of an LP precursor (A/chicken/Germany/AR915/2015 H7N7, AR915) and its HP descendant (A/chicken/Germany/AR1385/2015 H7N7, AR1385), differing, outside the HACS, by only very few mutations[19]. Infectivity titers are expressed as mean embryo infectious doses (EID_{50}/mL) using isolates at passage level 2 in ECEs.

Experimental design

In vivo co-infection experiment

In total, 168 white leghorn chickens, hatched from SPF ECEs (Lohmann Animal Health, Cuxhaven, Germany), were randomly assigned to 12 groups of 14 birds each at 6 weeks of age. Groups were housed in separate animal rooms. Each group tested AI-negative by serological and virological means (see below). Six groups (group M1-M6) were used to titrate clinical and pathohistological effects of the H7N7 HPAIV isolate AR1385 at doses of 10^1 (group M1), 10^3 (group M3), 10^4 (group M4), 10^5 (group M5), $10^{5.7}$ (group M5.7), or 10^6 (group M6) EID_{50} in 0.5 mL inoculum per bird. Another five groups (group C1-C5.7) received mixtures of a constant dose of H7N7 LP (10^6 EID_{50}) and HP viruses at different concentrations as shown in Table 1a. Finally, one group (B) received 10^6 EID_{50} in 0.5 mL per bird of the LPAIV H7N7 AR915 as a mono-infection. Oculo-oronasal inoculation mimicked a natural infection route in 10 chickens per group; four further chickens served as sentinels and were associated on dpi 1.

Clinical score and survival rate

During the study period of 13 days, chickens were monitored and scored threetimes a day for clinical signs: 0

Table 1a EID$_{50}$/0.5 mL per chicken used for the in vivo experiment

Group	EID$_{50}$/0.5 mL					
	10^1	10^3	10^4	10^5	$10^{5.7}$	10^6
C (+10^6 LP)	C1	C3	C4	C5	C5.7	–
M	M1	M3	M4	M5	M5.7	M6
B	–	–	–	–	–	B

Experimental design (classification of the groups), 1a in vivo, 1b in ovo experiment. EID$_{50}$ values refer to the HPAIV infection doses (groups C and M) and the LPAIV mono-infection dose (group B), respectively

Table 1b EID$_{50}$/0.2 mL per egg used for the in ovo experiment

Group	EID$_{50}$/0.2 mL					
	10^1	10^3	10^4	10^5	$10^{5.7}$	10^6
C (+10^6 LP)	C1	C3	C4	C5	C5.7	–
M	M1	M3	M4	M5	M5.7	M6
B	–	–	–	–	–	B

C = co-infection, M = mono-infection, B = mono LPAIV-infection

(normal/healthy), 1 (sick), 2 (severely sick), or 3 (dead). The highest score obtained at each day for a bird was used for statistical comparisons. Sick chickens showed one of the following symptoms: mild depression/tiredness and ruffled feathers, mild respiratory manifestations, facial edema, tentative feed intake or mild neurological signs. Severely sick birds showed two or more signs as described above and, in addition, cyanosis of the comb and the wattles, diarrhea, severe neurological signs (such as paralysis or convulsions). Moribund chickens reaching humane termination criteria were permanently drowsy and recumbent, could not be urged to move or showed severe dyspneic movement of the sternum. Such birds were humanely killed and registered as "3" (=dead) the day after. Morbidity and mortality indices were calculated according to the O.I.E. regulations for the Intravenous Pathogenicity Index (IVPI)[10].

At dpi 2, four chickens per group (each two inoculated and two sentinel chickens) were sacrificed in deep isoflurane anesthesia and subjected to postmortem examination. These animals were not considered for mortality index calculations. At dpi 13, surviving chickens were finally bled.

Sampling strategy

Serum samples were taken before virus exposure (dpi 0) and at 2 (birds sacrificed for histopathology), 6, and 13 dpi. Oropharyngeal (OP) and cloacal (CL) swabs to be collected from all virus-inoculated as well as all sentinel chickens were scheduled for dpi 0, 1, 2, 4, 6, 9, and 13 to assess virus shedding via the respiratory and digestive tracts. Birds were also swabbed when found dead or when they met the termination criteria. OP and CL swab samples were collected in 1 mL of serum-free cell culture medium and kept cooled at 4 °C until processed within 2 h after collection. Remaining swab supernatant was kept frozen at −70 °C.

In ovo experiment

A similar co-infection experiment of LP AR915 and HP AR1385 H7N7 viruses was carried out in ovo using two series of 10-day old and 14-day old SPF ECEs. Infection of five eggs per group was done by the allantoic route according to standard protocols[10,47,57]. Inoculation doses of mono-infected and co-infected ECEs (groups M1–5.7 and C1–5.7 and B) are shown in Table 1b. Eggs were incubated at 37 °C and candled daily for embryonic vitality. After embryonic death or a maximum of 96 h of incubation eggs were chilled at 4 °C for a minimum of 4 h. Amnio-allantoic fluids (AAFs) were harvested and assayed for HA as described[10]. Only eggs that were hemagglutination-positive in the AAF were considered for mean-death-time (MDT) calculations. Eggs with no HA titer (i.e., "no AIV-related death") or which did not die within 4 days scored a value of 120 h post inoculation (hpi). In addition, RNA extracted from AAF was tested by RT-qPCR as described below.

Furthermore, the embryo and the allantoic epithelium of two of the five eggs was dissected following the techniques described by Seekings[58] and immersed in 10% neutral buffered formalin for histopathological analysis. Further embryonic tissues (brain, liver, and heart) of two eggs per group were sampled for RT-qPCR analysis. Virus present in AAF that was in contact with the embryo's skin was removed by washing the embryos repeatedly and extensively in phosphate-buffered saline before incision for tissue removal.

Detection of viral shedding and molecular pathotyping of AIV RNA

Viral RNA was extracted from swab fluids (chickens), AAF and tissue samples (ECE) using the NucleoMag®VET Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions and stored at −20 °C until use. By using quantitative real-time RT-PCR (RT-qPCR), presence of RNA of the influenza A virus matrix (M) gene was confirmed by the protocol of Hoffmann, et al.[59]. M-positive samples were further subtyped by H7 pathotype-specific RT-qPCRs, which allowed probe-assisted differentiation of the mono-basic and polybasic HACS of the LP and HP H7 pathotype[60]. All RT-qPCR reactions were performed in 25 µL volumes using the

AgPath-ID RT-PCR kit (Ambion, Austin, TX, USA) and run on a CFX96 thermocycler machine (Bio-Rad).

Serology

After heat inactivation for 120 min at 56 °C (safety precautions), all sera were examined for antibodies against the AIV nucleoprotein (NP) using an Influenza A Antibody Competition enzyme-linked-immunosorbent assay (ELISA) (ID Screen®, IDVET, Grabels, France) according to the manufacturer's recommendations.

Histopathology and immunohistochemistry

Selected tissues and three cross sections of the skull (nasal cavity and paranasal sinuses) from chickens sacrificed at 2 dpi as well as tissue samples from the in ovo experiments were fixed in 10% neutral buffered formalin, and processed for hematoxylin and eosin staining. The severity of necrotizing inflammation, epithelial degeneration and/or necrosis in the nasal mucosa, as well as lymphatic necrosis, apoptosis and/or depletion in the lymphatic organs was scored on an ordinal 4-step scale (0 = unchanged, 1 = mild, 2 = moderate, 3 = severe).

IHC was performed on serial sections to detect influenza A virus antigen using the avidin-biotin-peroxidase complex method (Vectastain PK 6100; Vector Laboratories, Burlingame, CA, USA) with citric buffer (10 mM, pH 6.0) pretreatment. Antigen detection was achieved with a monoclonal antibody (mAb) directed against an epitope of the influenza A matrixprotein (ATCC clone HB-64). 3-amino-9-ethylcarbazol served as the chromogen (Agilent Technologies, Santa Clara, CA, USA), and hematoxylin counterstaining. Validated positive and negative archival tissues, as well as replacement of the specific antibody by Tris-Buffered-Saline (TBS) served as controls. The distribution of parenchymal, as well as endothelial influenza A matrixprotein was evaluated on an ordinal 4-step scale (0 = none, 1 = focal/oligofocal, 2 = multifocal, 3 = coalescing/diffuse).

Statistical analyses

The Mantel-Haenszel logrank test and the Mann Whitney test were used to compare survival rates and morbidity index as well as MDT values, respectively, applying the R software environment and the following packages: "stats", "survival", "survminer", "gridExtra" and "ggplot2". P values < 0.05 were considered significant. For comparisons between the total amount of virus shedding of HPAIV in the mono-infected and co-infected groups, area-under-the-curvegraphs were computed by using R software packages "stats", "survival", "survminer", and "ggplot2". The mean average of Cq values of all animals sampled at the indicated dpi in a specific group were calculated and used to draw the curves. Animals negative in RT-qPCR at that date scored with a value of 40.

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Author contribution

M.B, T.C.M., and T.C.H. devised the project and the main conceptual ideas. A.G., D.S., S.K., and T.C.H. carried out the infection experiments. R.U. and A.G. performed the pathological and histopathological investigations. A.G. and T.C. H. processed the experimental data, performed the initial analysis, drafted the manuscript with input from E.A. and designed the figures. P.M. carried out the statistical analyses and generated the figures. All authors contributed to the interpretation of the results. All authors approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplemental material

Supplemental Table 1a. P-values of clinical scores (supplement to Figure 1a).

Group	infected	sentinel
C1 vs M1	NA	0.331753367
C3 vs. M3	5.58E-09	0.066627494
C4 vs. M4	2.35E-05	0.290733828
C5 vs. M5	0.8287837	0.333533006
C5.7 vs. M5.7	0.2372944	0.886651032
B vs. M6	2.78E-11	0.00038952

Supplemental Table 1b. P-values of survival probabilities (supplement to Figure 1b).

Group	infected	sentinel
C1 vs M1	1	1
C3 vs. M3	4.16E-07	0.0000374
C4 vs. M4	2.08E-03	0.0000585
C5 vs. M5	0.000666797	0.1631753
C5.7 vs. M5.7	0.037529348	0.0101169
B vs. M6	3.22E-18	0.0000527

Supplemental Table 2. RT-qPCR results of *in vivo* experiments (supplement to Figure 1c).

group C1				Real-time RT-PCR					
	dpi	status	animal ID	M1.2 OP	M1.2 CL	H7 LP OP	H7 LP CL	H7 HP OP	H7 HP CL
1	1	inoculated	C1-1	29.14	neg	30.72	neg	neg	neg
2	1	inoculated	C1-2	33.51	neg	neg	neg	neg	neg
3	1	inoculated	C1-3	28.74	neg	31.55	neg	neg	neg
4	1	inoculated	C1-4	26.12	neg	28.06	neg	neg	neg
5	1	inoculated	C1-5	35.54	neg	neg	neg	neg	neg
6	1	inoculated	C1-6	33.07	neg	35.99	neg	neg	neg
7	1	inoculated	C1-7	34.48	neg	neg	neg	neg	neg
8	1	inoculated	C1-8	31.06	neg	37.61	neg	neg	neg
9	1	inoculated	C1-9	38.36	neg	neg	neg	neg	neg
10	1	inoculated	C1-10	30.30	neg	32.11	neg	neg	neg
11	1	sentinel	C1-11	neg	neg	neg	neg	neg	neg
12	1	sentinel	C1-12	neg	neg	neg	neg	neg	neg
13	1	sentinel	C1-13	neg	neg	neg	neg	neg	neg
14	1	sentinel	C1-14	36.48	neg	neg	neg	neg	neg
15	2	inoculated	C1-1	31.94	neg	32.85	neg	neg	neg
16	2	inoculated	C1-2	31.23	neg	33.55	neg	neg	neg
17	2	inoculated	C1-3	32.14	39.71	32.42	neg	neg	neg
18	2	inoculated	C1-4	29.05	neg	30.90	neg	neg	neg
19	2	inoculated	C1-5	32.21	neg	33.67	neg	neg	neg
20	2	inoculated	C1-6	31.76	neg	33.19	neg	neg	neg
21	2	inoculated	C1-7	33.19	neg	34.90	neg	neg	neg
22	2	inoculated	C1-8	31.11	neg	34.07	neg	neg	neg
23	2	inoculated	C1-9	neg	neg	neg	neg	neg	neg
24	2	inoculated	C1-10	36.42	neg	neg	neg	neg	neg
25	2	sentinel	C1-11	neg	neg	neg	neg	neg	neg
26	2	sentinel	C1-12	neg	neg	neg	neg	neg	neg
27	2	sentinel	C1-13	neg	neg	neg	neg	neg	neg
28	2	sentinel	C1-14	neg	neg	neg	neg	neg	neg
30	4	inoculated	C1-2	34.59	neg	neg	neg	neg	neg
31	4	inoculated	C1-3	32.35	neg	neg	neg	neg	neg
32	4	inoculated	C1-4	34.11	28.11	neg	neg	neg	neg
33	4	inoculated	C1-5	28.12	36.32	30.66	neg	neg	neg
34	4	inoculated	C1-6	29.08	37.20	35.08	neg	neg	neg
35	4	inoculated	C1-7	36.78	neg	neg	neg	neg	neg
36	4	inoculated	C1-8	neg	neg	neg	neg	neg	neg
37	4	inoculated	C1-9	neg	neg	neg	neg	neg	neg
38	4	inoculated	C1-10	neg	neg	neg	neg	neg	neg
41	4	sentinel	C1-13	38.09	37.07	neg	neg	neg	neg
44	6	inoculated	C1-2	35.17	36.66	neg	neg	neg	neg
45	6	inoculated	C1-3	37.45	28.20	neg	30.47	neg	neg
46	6	inoculated	C1-4	34.66	neg	neg	neg	neg	neg
47	6	inoculated	C1-5	34.06	35.20	neg	37.24	neg	neg
48	6	inoculated	C1-6	neg	32.00	neg	neg	neg	neg
49	6	inoculated	C1-7	neg	25.06	neg	31.38	neg	neg
50	6	inoculated	C1-8	37.52	33.24	35.82	neg	neg	neg
51	6	inoculated	C1-9	neg	neg	neg	neg	neg	neg
53	6	sentinel	C1-10	neg	37.13	neg	neg	neg	neg
55	6	sentinel	C1-13	27.85	neg	32.00	neg	neg	neg
58	9	inoculated	C1-2	neg	39.51	neg	neg	neg	neg
59	9	inoculated	C1-3	38.66	33.10	neg	34.89	neg	neg
60	9	inoculated	C1-4	neg	35.24	neg	neg	neg	neg
61	9	inoculated	C1-5	neg	37.64	neg	neg	neg	neg
62	9	inoculated	C1-6	neg	34.89	neg	neg	neg	neg
63	9	inoculated	C1-7	neg	34.55	neg	neg	neg	neg
64	9	inoculated	C1-8	neg	32.66	neg	33.25	neg	neg
65	9	inoculated	C1-9	neg	33.87	neg	neg	neg	neg
68	9	sentinel	C1-10	neg	32.75	neg	neg	neg	neg
69	9	sentinel	C1-13	31.12	31.16	36.05	neg	neg	neg
72	13	inoculated	C1-2	neg	neg	neg	neg	neg	neg
73	13	inoculated	C1-3	neg	neg	neg	neg	neg	neg
74	13	inoculated	C1-4	neg	neg	neg	neg	neg	neg
75	13	inoculated	C1-5	neg	neg	neg	neg	neg	neg
76	13	inoculated	C1-6	neg	neg	neg	neg	neg	neg
77	13	inoculated	C1-7	neg	32.07	neg	neg	neg	neg
78	13	inoculated	C1-8	neg	neg	neg	neg	neg	neg
79	13	inoculated	C1-9	neg	neg	neg	neg	neg	neg
82	13	sentinel	C1-10	neg	neg	neg	neg	neg	neg
83	13	sentinel	C1-13	neg	32.16	neg	36.84	neg	neg

group C3				Real-time RT-PCR					
	dpi	status	animal ID	M1.2 OP	M1.2 CL	H7 LP OP	H7 LP CL	H7 HP OP	H7 HP CL
1	1	inoculated	C3-1	32.39	neg	35.10	neg	neg	neg
2	1	inoculated	C3-2	30.04	neg	32.86	neg	neg	neg
3	1	inoculated	C3-3	32.03	neg	36.32	neg	neg	neg
4	1	inoculated	C3-4	neg	neg	neg	neg	neg	neg
5	1	inoculated	C3-5	27.66	neg	31.80	neg	neg	neg
6	1	inoculated	C3-6	28.42	neg	31.09	neg	neg	neg
7	1	inoculated	C3-7	24.03	neg	27.29	neg	neg	neg
8	1	inoculated	C3-8	26.45	36.65	29.80	neg	neg	neg
9	1	inoculated	C3-9	23.39	35.03	26.67	neg	neg	neg
10	1	inoculated	C3-10	34.45	neg	neg	neg	neg	neg
11	1	sentinel	C3-11	neg	neg	neg	neg	neg	neg
12	1	sentinel	C3-12	neg	neg	neg	neg	neg	neg
13	1	sentinel	C3-13	neg	neg	neg	neg	neg	neg
14	1	sentinel	C3-14	neg	neg	neg	neg	neg	neg
15	2	inoculated	C3-1	28.51	36.06	30.82	neg	neg	neg
16	2	inoculated	C3-2	31.24	neg	30.24	neg	neg	neg
17	2	inoculated	C3-3	29.77	neg	31.80	neg	neg	neg
18	2	inoculated	C3-4	31.28	35.70	34.02	neg	neg	neg
19	2	inoculated	C3-5	29.64	neg	33.02	neg	neg	neg
20	2	inoculated	C3-6	29.63	neg	31.75	neg	neg	neg
21	2	inoculated	C3-7	30.96	32.26	33.62	neg	neg	neg
22	2	inoculated	C3-8	31.99	21.13	neg	23.85	neg	neg
23	2	inoculated	C3-9	30.30	38.88	33.91	neg	neg	neg
24	2	inoculated	C3-10	31.66	neg	34.66	neg	neg	neg
25	2	sentinel	C3-11	38.46	neg	neg	neg	neg	neg
26	2	sentinel	C3-12	38.22	neg	neg	neg	neg	neg
27	2	sentinel	C3-13	33.91	neg	neg	neg	neg	neg
28	2	sentinel	C3-14	31.02	38.23	34.70	neg	neg	neg
29	4	inoculated	C3-1	39.02	neg	neg	neg	neg	neg
30	4	inoculated	C3-2	neg	38.63	neg	36.51	neg	neg
32	4	inoculated	C3-4	36.95	neg	neg	neg	neg	neg
33	4	inoculated	C3-5	27.22	neg	30.79	neg	neg	neg
34	4	inoculated	C3-6	30.83	35.46	30.27	neg	neg	neg
35	4	inoculated	C3-7	31.85	38.25	35.80	neg	neg	neg
37	4	inoculated	C3-9	27.55	30.85	31.39	neg	neg	neg
38	4	inoculated	C3-10	26.26	31.02	30.16	neg	neg	neg
39	4	sentinel	C3-11	34.42	27.13	neg	neg	neg	neg
42	4	sentinel	C3-14	neg	neg	neg	neg	neg	neg
43	6	inoculated	C3-1	38.92	30.81	neg	31.91	neg	neg
44	6	inoculated	C3-2	33.37	neg	37.08	neg	neg	neg
46	6	inoculated	C3-4	29.11	neg	31.99	neg	neg	neg
47	6	inoculated	C3-5	35.30	neg	neg	neg	neg	neg
48	6	inoculated	C3-6	27.27	35.45	30.12	neg	neg	neg
49	6	inoculated	C3-7	35.14	22.78	neg	24.03	neg	neg
51	6	inoculated	C3-9	31.23	38.33	35.81	neg	neg	neg
52	6	inoculated	C3-10	37.43	36.51	36.09	36.32	neg	neg
53	6	sentinel	C3-11	33.31	neg	37.51	neg	neg	neg
56	6	sentinel	C3-14	38.19	29.17	neg	30.39	neg	neg
57	9	inoculated	C3-1	neg	24.17	neg	29.50	neg	neg
58	9	inoculated	C3-2	neg	38.00	neg	neg	neg	neg
60	9	inoculated	C3-4	35.20	37.99	neg	neg	neg	neg
61	9	inoculated	C3-5	neg	38.03	neg	neg	neg	neg
62	9	inoculated	C3-6	36.29	neg	38.99	neg	neg	neg
63	9	inoculated	C3-7	neg	35.00	neg	neg	neg	neg
65	9	inoculated	C3-9	35.68	32.73	neg	37.46	neg	neg
66	9	inoculated	C3-10	neg	37.52	neg	neg	neg	neg
67	9	sentinel	C3-11	36.27	neg	neg	neg	neg	neg
70	9	sentinel	C3-14	neg	32.61	neg	38.16	neg	neg
71	13	inoculated	C3-1	neg	33.57	neg	neg	neg	neg
72	13	inoculated	C3-2	34.83	neg	35.31	neg	neg	neg
74	13	inoculated	C3-4	37.86	37.80	neg	neg	neg	neg
75	13	inoculated	C3-5	neg	neg	neg	neg	neg	neg
76	13	inoculated	C3-6	35.95	neg	neg	neg	neg	neg
77	13	inoculated	C3-7	neg	34.38	neg	neg	neg	neg
79	13	inoculated	C3-9	neg	37.87	neg	neg	neg	neg
80	13	inoculated	C3-10	neg	neg	neg	neg	neg	neg
81	13	sentinel	C3-11	neg	neg	neg	neg	neg	neg
82	13	sentinel	C3-14	neg	31.80	neg	33.46	neg	neg

group C4				Real-time RT-PCR					
	dpi	status	animal ID	M1.2 OP	M1.2 CL	H7 LP OP	H7 LP CL	H7 HP OP	H7 HP CL
1	1	inoculated	C4-1	24.51	neg	30.15	neg	28.36	neg
2	1	inoculated	C4-2	20.65	neg	23.44	neg	neg	neg
3	1	inoculated	C4-3	23.66	neg	27.28	neg	neg	neg
4	1	inoculated	C4-4	26.50	neg	29.56	neg	neg	neg
5	1	inoculated	C4-5	26.07	neg	29.49	neg	neg	neg
6	1	inoculated	C4-6	23.88	35.79	25.75	neg	neg	neg
7	1	inoculated	C4-7	26.08	neg	28.70	neg	neg	neg
8	1	inoculated	C4-8	26.43	neg	29.86	neg	neg	neg
9	1	inoculated	C4-9	33.63	neg	neg	neg	neg	neg
10	1	inoculated	C4-10	25.34	neg	27.13	neg	neg	neg
11	1	sentinel	C4-11	neg	neg	neg	neg	neg	neg
12	1	sentinel	C4-12	neg	neg	neg	neg	neg	neg
13	1	sentinel	C4-13	neg	neg	neg	neg	neg	neg
14	1	sentinel	C4-14	neg	neg	neg	neg	neg	neg
15	2	inoculated	C4-1	28.41	neg	neg	neg	29.29	neg
16	2	inoculated	C4-2	30.11	neg	30.27	30.86	neg	neg
17	2	inoculated	C4-3	29.22	neg	30.35	neg	neg	neg
18	2	inoculated	C4-4	30.04	37.18	32.42	neg	neg	neg
19	2	inoculated	C4-5	31.69	22.02	neg	neg	neg	27.29
20	2	inoculated	C4-6	32.46	neg	neg	neg	neg	neg
21	2	inoculated	C4-7	28.19	neg	30.36	neg	neg	neg
22	2	inoculated	C4-8	27.55	neg	29.28	neg	neg	neg
23	2	inoculated	C4-9	34.15	neg	neg	neg	neg	neg
24	2	inoculated	C4-10	26.32	37.41	28.07	neg	neg	neg
25	2	sentinel	C4-11	35.64	neg	neg	neg	neg	neg
26	2	sentinel	C4-12	neg	neg	neg	neg	neg	neg
27	2	sentinel	C4-13	neg	neg	neg	neg	neg	neg
28	2	sentinel	C4-14	29.63	neg	33.89	neg	neg	neg
29	4	inoculated	C4-1	neg	24.23	neg	neg	neg	30.31
30	4	inoculated	C4-2	38.10	35.09	neg	30.37	33.24	neg
31	4	inoculated	C4-3	35.25	35.63	36.07	neg	32.34	34.35
32	4	inoculated	C4-4	neg	neg	neg	neg	neg	neg
33	4	inoculated	C4-5	33.07	36.27	37.03	neg	neg	neg
34	4	inoculated	C4-8	36.42	32.77	31.78	neg	neg	neg
35	4	inoculated	C4-9	29.38	25.97	neg	neg	neg	neg
36	4	inoculated	C4-10	23.03	29.12	25.44	27.17	neg	neg
37	4	sentinel	C4-11	33.13	neg	neg	neg	neg	neg
38	4	sentinel	C4-14	27.20	22.54	neg	neg	27.99	neg
39	5	inoculated	C4-3	26.56	26.45	neg	neg	23.40	24.96
40	6	inoculated	C4-2	33.30	26.04	neg	27.33	neg	neg
41	6	inoculated	C4-4	34.60	38.32	37.53	neg	neg	neg
42	6	inoculated	C4-5	33.47	35.69	37.01	neg	neg	neg
43	6	inoculated	C4-8	33.47	33.82	37.07	neg	neg	neg
44	6	inoculated	C4-9	neg	neg	neg	neg	neg	neg
45	6	inoculated	C4-10	36.33	32.02	36.26	33.93	neg	neg
46	6	sentinel	C4-11	36.20	22.72	neg	25.94	neg	neg
47	6	sentinel	C4-14	35.78	27.06	neg	30.10	neg	neg
48	9	inoculated	C4-2	neg	32.18	neg	34.75	neg	neg
50	9	inoculated	C4-4	38.24	38.17	neg	neg	neg	neg
51	9	inoculated	C4-5	neg	neg	neg	neg	neg	neg
52	9	inoculated	C4-8	neg	39.93	neg	neg	neg	neg
53	9	inoculated	C4-9	36.88	neg	neg	neg	neg	neg
54	9	sentinel	C4-10	34.40	32.50	neg	37.80	neg	neg
55	9	sentinel	C4-11	35.64	30.24	neg	36.10	neg	neg
56	9	sentinel	C4-14	neg	31.81	neg	35.01	neg	neg
57	13	inoculated	C4-2	neg	33.13	neg	34.68	neg	neg
58	13	inoculated	C4-4	neg	37.92	neg	neg	neg	neg
59	13	inoculated	C4-5	neg	neg	neg	neg	neg	neg
60	13	inoculated	C4-8	neg	39.15	neg	neg	neg	neg
61	13	inoculated	C4-9	neg	neg	neg	neg	neg	neg
62	13	sentinel	C4-10	neg	31.04	neg	37.74	neg	neg
63	13	sentinel	C4-11	37.90	34.39	neg	neg	neg	neg
64	13	sentinel	C4-14	neg	38.33	neg	neg	neg	neg

group C5				Real-time RT-PCR					
	dpi	status	animal ID	M1.2 OP	M1.2 CL	H7 LP OP	H7 LP CL	H7 HP OP	H7 HP CL
1	1	inoculated	C5-1	23.54	36.09	27.67	neg	28.02	neg
2	1	inoculated	C5-2	25.23	neg	30.44	neg	28.71	neg
3	1	inoculated	C5-3	23.08	35.12	28.94	neg	23.79	neg
4	1	inoculated	C5-4	28.20	neg	neg	neg	29.48	neg
5	1	inoculated	C5-5	25.02	neg	32.15	neg	25.87	neg
6	1	inoculated	C5-6	25.61	neg	36.91	neg	26.62	neg
7	1	inoculated	C5-7	24.48	neg	28.94	neg	25.66	neg
8	1	inoculated	C5-8	25.01	38.78	neg	neg	26.67	neg
9	1	inoculated	C5-9	24.30	neg	30.85	neg	25.43	neg
10	1	inoculated	C5-10	24.56	neg	34.27	neg	25.98	neg
11	1	sentinel	C5-11	neg	neg	neg	neg	neg	neg
12	1	sentinel	C5-12	neg	neg	neg	neg	neg	neg
13	1	sentinel	C5-13	neg	neg	neg	neg	neg	neg
14	1	sentinel	C5-14	neg	neg	neg	neg	neg	neg
15	2	inoculated	C5-1	30.11	28.38	neg	28.80	neg	neg
16	2	inoculated	C5-2	27.52	37.03	35.66	35.32	30.42	neg
17	2	inoculated	C5-3	27.54	29.76	neg	neg	29.78	35.16
18	2	inoculated	C5-4	29.20	33.24	neg	neg	32.09	neg
19	2	inoculated	C5-5	29.87	27.02	35.44	neg	neg	28.61
20	2	inoculated	C5-6	29.64	37.07	neg	neg	32.78	neg
21	2	inoculated	C5-7	31.50	22.61	neg	27.48	32.35	22.91
22	2	inoculated	C5-8	29.53	26.89	neg	neg	33.10	27.59
23	2	inoculated	C5-9	30.34	35.58	neg	neg	32.39	neg
24	2	inoculated	C5-10	28.24	31.97	neg	neg	29.66	neg
25	2	sentinel	C5-11	38.15	neg	neg	neg	neg	neg
26	2	sentinel	C5-12	34.54	neg	neg	neg	neg	neg
27	2	sentinel	C5-13	35.83	neg	neg	neg	neg	neg
28	2	sentinel	C5-14	38.47	neg	neg	neg	neg	neg
29	3	inoculated	C5-3	28.65	29.32	neg	neg	30.60	32.54
31	4	inoculated	C5-1	24.71	26.34	29.17	neg	24.58	27.77
32	4	inoculated	C5-2	24.85	26.86	31.39	neg	30.09	30.19
33	4	inoculated	C5-5	24.28	neg	31.38	neg	neg	neg
34	4	inoculated	C5-6	neg	28.76	neg	30.31	neg	25.41
35	4	inoculated	C5-8	28.50	27.90	31.02	neg	27.23	26.11
36	4	inoculated	C5-9	25.26	24.42	32.06	neg	26.44	29.78
37	4	inoculated	C5-10	25.14	23.69	31.39	neg	28.94	30.09
38	4	inoculated	C5-11	28.94	neg	34.33	neg	neg	neg
39	4	sentinel	C5-13	23.95	31.56	33.06	neg	38.00	33.30
41	5	inoculated	C5-1	24.71	27.65	27.87	29.79	20.97	25.55
42	5	inoculated	C5-2	24.85	25.43	28.30	neg	24.60	28.76
43	5	inoculated	C5-8	31.09	29.17	31.38	neg	neg	29.69
44	5	inoculated	C5-9	neg	31.39	neg	neg	24.89	25.42
45	5	inoculated	C5-10	25.14	34.35	neg	neg	24.26	29.50
46	5	inoculated	C5-11	30.02	neg	31.83	neg	35.51	neg
47	5	sentinel	C5-13	23.95	neg	33.06	neg	neg	neg
48	6	sentinel	C5-11	30.71	34.33	neg	neg	30.75	35.26

group C5.7				Real-time RT-PCR					
	dpi	status	animal ID	M1.2 OP	M1.2 CL	H7 LP OP	H7 LP CL	H7 HP OP	H7 HP CL
1	1	inoculated	C5.7-1	23.16	36.77	neg	neg	24.37	neg
2	1	inoculated	C5.7-2	24.72	neg	33.32	neg	25.21	neg
3	1	inoculated	C5.7-3	25.61	neg	33.86	neg	26.37	neg
4	1	inoculated	C5.7-4	29.10	neg	36.15	neg	neg	neg
5	1	inoculated	C5.7-5	23.42	neg	neg	neg	24.56	neg
6	1	inoculated	C5.7-6	25.39	neg	neg	neg	26.29	neg
7	1	inoculated	C5.7-7	26.57	39.75	neg	neg	27.46	neg
8	1	inoculated	C5.7-8	28.62	38.78	neg	neg	30.09	neg
9	1	inoculated	C5.7-9	25.28	neg	36.21	neg	26.38	neg
10	1	inoculated	C5.7-10	22.15	neg	neg	neg	22.17	neg
11	1	sentinel	C5.7-11	37.73	neg	neg	neg	neg	neg
12	1	sentinel	C5.7-12	neg	27.18	neg	32.80	neg	neg
13	1	sentinel	C5.7-13	neg	neg	neg	neg	neg	neg
14	1	sentinel	C5.7-14	neg	neg	neg	neg	neg	neg
15	2	inoculated	C5.7-1	28.64	neg	neg	neg	29.05	35.36
16	2	inoculated	C5.7-2	29.26	32.98	neg	neg	29.65	neg
17	2	inoculated	C5.7-3	28.22	37.08	neg	neg	29.08	neg
18	2	inoculated	C5.7-4	28.41	26.37	36.73	neg	35.34	neg
19	2	inoculated	C5.7-5	26.75	20.21	neg	neg	27.99	neg
20	2	inoculated	C5.7-6	30.35	35.99	neg	neg	35.77	neg
21	2	inoculated	C5.7-7	28.13	35.44	neg	neg	29.66	neg
22	2	inoculated	C5.7-8	29.09	34.09	neg	neg	29.38	21.47
23	2	inoculated	C5.7-9	31.80	34.36	neg	neg	34.84	28.13
24	2	inoculated	C5.7-10	33.24	34.17	neg	neg	neg	neg
25	2	sentinel	C5.7-11	32.26	31.00	neg	neg	neg	neg
26	2	sentinel	C5.7-12	neg	28.38	neg	neg	neg	neg
27	2	sentinel	C5.7-13	neg	31.53	neg	neg	neg	neg
28	2	sentinel	C5.7-14	neg	neg	neg	neg	neg	neg
29	4	inoculated	C5.7-1	23.28	19.29	neg	neg	24.19	20.54
30	4	inoculated	C5.7-3	23.98	24.82	neg	neg	24.42	24.48
31	4	inoculated	C5.7-4	25.39	24.46	neg	neg	25.62	25.23
32	4	inoculated	C5.7-5	22.51	23.13	neg	neg	24.01	24.63
33	4	inoculated	C5.7-7	25.46	26.38	neg	37.46	26.06	28.14
34	4	inoculated	C5.7-8	22.67	24.78	neg	neg	23.32	26.43
35	4	inoculated	C5.7-9	21.10	24.39	38.20	neg	21.81	25.22
36	4	inoculated	C5.7-10	28.16	29.33	neg	neg	28.39	29.22
37	4	sentinel	C5.7-11	29.29	neg	neg	neg	29.86	neg
38	4	sentinel	C5.7-12	29.25	37.34	35.31	neg	31.09	21.74
39	5	inoculated	C5.7-7	29.22	33.04	neg	neg	22.47	23.61
40	5	inoculated	C5.7-10	24.27	23.14	neg	neg	27.40	22.76
41	6	sentinel	C5.7-11	27.27	25.51	neg	neg	29.40	29.73
42	6	sentinel	C5.7-12	21.56	25.82	neg	neg	24.50	26.02
43	7	sentinel	C5.7-11	26.26	25.49	neg	neg	28.68	26.74

group M1				Real-time RT-PCR					
	dpi	status	animal ID	M1.2 OP	M1.2 CL	H7 LP OP	H7 LP CL	H7 HP OP	H7 HP CL
1	1	inoculated	M1-1	neg	neg	neg	neg	neg	neg
2	1	inoculated	M1-2	neg	neg	neg	neg	neg	neg
3	1	inoculated	M1-3	neg	neg	neg	neg	neg	neg
4	1	inoculated	M1-4	neg	neg	neg	neg	neg	neg
5	1	inoculated	M1-5	neg	neg	neg	neg	neg	neg
6	1	inoculated	M1-6	neg	neg	neg	neg	neg	neg
7	1	inoculated	M1-7	neg	neg	neg	neg	neg	neg
8	1	inoculated	M1-8	neg	neg	neg	neg	neg	neg
9	1	inoculated	M1-9	neg	neg	neg	neg	neg	neg
10	1	inoculated	M1-10	neg	neg	neg	neg	neg	neg
11	1	sentinel	M1-11	neg	neg	neg	neg	neg	neg
12	1	sentinel	M1-12	neg	neg	neg	neg	neg	neg
13	1	sentinel	M1-13	neg	neg	neg	neg	neg	neg
14	1	sentinel	M1-14	neg	neg	neg	neg	neg	neg
15	2	inoculated	M1-1	neg	neg	neg	neg	neg	neg
16	2	inoculated	M1-2	neg	neg	neg	neg	neg	neg
17	2	inoculated	M1-3	neg	neg	neg	neg	neg	neg
18	2	inoculated	M1-4	neg	neg	neg	neg	neg	neg
19	2	inoculated	M1-5	neg	neg	neg	neg	neg	neg
20	2	inoculated	M1-6	neg	neg	neg	neg	neg	neg
21	2	inoculated	M1-7	neg	neg	neg	neg	neg	neg
22	2	inoculated	M1-8	neg	neg	neg	neg	neg	neg
23	2	inoculated	M1-9	neg	neg	neg	neg	neg	neg
24	2	inoculated	M1-10	neg	neg	neg	neg	neg	neg
25	2	sentinel	M1-11	neg	neg	neg	neg	neg	neg
26	2	sentinel	M1-12	neg	neg	neg	neg	neg	neg
27	2	sentinel	M1-13	neg	neg	neg	neg	neg	neg
28	2	sentinel	M1-14	neg	neg	neg	neg	neg	neg
29	4	inoculated	M1-1	neg	neg	neg	neg	neg	neg
30	4	inoculated	M1-2	neg	neg	neg	neg	neg	neg
31	4	inoculated	M1-3	neg	neg	neg	neg	neg	neg
32	4	inoculated	M1-4	neg	neg	neg	neg	neg	neg
33	4	inoculated	M1-5	neg	neg	neg	neg	neg	neg
34	4	inoculated	M1-6	neg	neg	neg	neg	neg	neg
35	4	inoculated	M1-9	neg	neg	neg	neg	neg	neg
36	4	inoculated	M1-10	neg	neg	neg	neg	neg	neg
37	4	sentinel	M1-11	neg	neg	neg	neg	neg	neg
38	4	sentinel	M1-12	neg	neg	neg	neg	neg	neg
39	6	inoculated	M1-1	neg	neg	neg	neg	neg	neg
40	6	inoculated	M1-2	neg	neg	neg	neg	neg	neg
41	6	inoculated	M1-3	neg	neg	neg	neg	neg	neg
42	6	inoculated	M1-4	neg	neg	neg	neg	neg	neg
43	6	inoculated	M1-5	neg	neg	neg	neg	neg	neg
44	6	inoculated	M1-6	neg	neg	neg	neg	neg	neg
45	6	inoculated	M1-9	neg	neg	neg	neg	neg	neg
46	6	inoculated	M1-10	neg	neg	neg	neg	neg	neg
47	6	sentinel	M1-11	neg	neg	neg	neg	neg	neg
48	6	sentinel	M1-12	neg	neg	neg	neg	neg	neg
49	9	inoculated	M1-1	neg	neg	neg	neg	neg	neg
50	9	inoculated	M1-2	neg	neg	neg	neg	neg	neg
51	9	inoculated	M1-3	neg	neg	neg	neg	neg	neg
52	9	inoculated	M1-4	neg	neg	neg	neg	neg	neg
53	9	inoculated	M1-5	neg	neg	neg	neg	neg	neg
54	9	inoculated	M1-6	neg	neg	neg	neg	neg	neg
55	9	inoculated	M1-9	neg	neg	neg	neg	neg	neg
56	9	inoculated	M1-10	neg	neg	neg	neg	neg	neg
57	9	sentinel	M1-11	neg	neg	neg	neg	neg	neg
58	9	sentinel	M1-12	neg	neg	neg	neg	neg	neg
59	13	inoculated	M1-1	neg	neg	neg	neg	neg	neg
60	13	inoculated	M1-2	neg	neg	neg	neg	neg	neg
61	13	inoculated	M1-3	neg	neg	neg	neg	neg	neg
62	13	inoculated	M1-4	neg	neg	neg	neg	neg	neg
63	13	inoculated	M1-5	neg	neg	neg	neg	neg	neg
64	13	inoculated	M1-6	neg	neg	neg	neg	neg	neg
65	13	inoculated	M1-9	neg	neg	neg	neg	neg	neg
66	13	inoculated	M1-10	neg	neg	neg	neg	neg	neg
67	13	sentinel	M1-11	neg	neg	neg	neg	neg	neg
68	13	sentinel	M1-12	neg	neg	neg	neg	neg	neg

group M3				Real-time RT-PCR					
	dpi	status	animal ID	M1.2 OP	M1.2 CL	H7 LP OP	H7 LP CL	H7 HP OP	H7 HP CL
1	1	inoculated	M3-1	neg	neg	neg	neg	neg	neg
2	1	inoculated	M3-2	neg	neg	neg	neg	neg	neg
3	1	inoculated	M3-3	neg	neg	neg	neg	neg	neg
4	1	inoculated	M3-4	neg	neg	neg	neg	neg	neg
5	1	inoculated	M3-5	neg	neg	neg	neg	neg	neg
6	1	inoculated	M3-6	neg	neg	neg	neg	neg	neg
7	1	inoculated	M3-7	neg	neg	neg	neg	neg	neg
8	1	inoculated	M3-8	neg	neg	neg	neg	neg	neg
9	1	inoculated	M3-9	38.36	neg	neg	neg	neg	neg
10	1	inoculated	M3-10	neg	neg	neg	neg	neg	neg
11	1	sentinel	M3-11	neg	neg	neg	neg	neg	neg
12	1	sentinel	M3-12	neg	neg	neg	neg	neg	neg
13	1	sentinel	M3-13	neg	neg	neg	neg	neg	neg
14	1	sentinel	M3-14	neg	neg	neg	neg	neg	neg
15	2	inoculated	M3-1	neg	neg	neg	neg	neg	neg
16	2	inoculated	M3-2	neg	neg	neg	neg	neg	neg
17	2	inoculated	M3-3	neg	neg	neg	neg	neg	neg
18	2	inoculated	M3-4	neg	neg	neg	neg	neg	neg
19	2	inoculated	M3-5	neg	neg	neg	neg	neg	neg
20	2	inoculated	M3-6	neg	neg	neg	neg	neg	neg
21	2	inoculated	M3-7	neg	neg	neg	neg	neg	neg
22	2	inoculated	M3-8	neg	neg	neg	neg	neg	neg
23	2	inoculated	M3-9	35.86	39.91	neg	neg	neg	neg
24	2	inoculated	M3-10	neg	neg	neg	neg	neg	neg
25	2	sentinel	M3-11	neg	neg	neg	neg	neg	neg
26	2	sentinel	M3-12	neg	neg	neg	neg	neg	neg
27	2	sentinel	M3-13	neg	neg	neg	neg	neg	neg
28	2	sentinel	M3-14	neg	neg	neg	neg	neg	neg
29	4	inoculated	M3-2	neg	neg	neg	neg	neg	neg
30	4	inoculated	M3-3	32.46	37.18	neg	neg	34.60	neg
31	4	inoculated	M3-4	38.37	neg	neg	neg	neg	neg
32	4	inoculated	M3-5	38.28	neg	neg	neg	neg	neg
33	4	inoculated	M3-7	33.25	neg	neg	neg	neg	neg
34	4	inoculated	M3-8	30.35	neg	neg	neg	30.08	neg
35	4	inoculated	M3-9	31.61	29.82	neg	neg	32.79	30.47
36	4	inoculated	M3-10	32.06	neg	neg	neg	neg	neg
37	4	sentinel	M3-11	38.40	neg	neg	neg	neg	neg
38	4	sentinel	M3-12	34.03	neg	neg	neg	neg	neg
39	6	inoculated	M3-2	34.49	neg	neg	neg	neg	neg
40	6	inoculated	M3-3	34.43	22.21	neg	neg	neg	22.73
41	6	inoculated	M3-4	36.05	31.84	neg	neg	neg	neg
42	6	inoculated	M3-5	30.96	35.11	neg	neg	34.02	neg
43	6	inoculated	M3-7	32.49	31.95	neg	neg	39.19	37.07
44	6	inoculated	M3-8	32.07	29.83	neg	neg	neg	31.62
45	6	inoculated	M3-9	28.16	20.35	neg	neg	28.49	21.55
46	6	inoculated	M3-10	32.43	25.97	neg	neg	37.30	27.03
47	6	sentinel	M3-11	24.62	26.30	neg	neg	24.39	27.44
48	6	sentinel	M3-12	27.21	24.53	neg	neg	28.06	24.82
49	7	inoculated	M3-9	25.93	23.07	neg	neg	28.21	24.19
50	8	inoculated	M3-3	27.29	26.35	neg	neg	27.36	27.15
51	8	inoculated	M3-5	24.25	26.85	neg	neg	25.18	29.00
52	8	inoculated	M3-7	26.72	24.44	neg	neg	27.54	25.40
53	8	inoculated	M3-8	24.94	28.02	neg	neg	24.51	29.23
54	8	inoculated	M3-10	25.86	28.56	neg	neg	26.18	31.29
55	9	inoculated	M3-2	37.53	neg	neg	neg	neg	neg
56	9	inoculated	M3-4	27.07	29.10	neg	neg	neg	29.18
57	10	inoculated	M3-2	36.95	neg	neg	neg	neg	neg

group M4				Real-time RT-PCR					
	dpi	status	animal ID	M1.2 OP	M1.2 CL	H7 LP OP	H7 LP CL	H7 HP OP	H7 HP CL
1	1	inoculated	M4-1	neg	neg	neg	neg	neg	neg
2	1	inoculated	M4-2	neg	neg	neg	neg	neg	neg
3	1	inoculated	M4-3	31.51	neg	neg	neg	35.61	neg
4	1	inoculated	M4-4	neg	neg	neg	neg	neg	neg
5	1	inoculated	M4-5	neg	neg	neg	neg	neg	neg
6	1	inoculated	M4-6	neg	neg	neg	neg	neg	neg
7	1	inoculated	M4-7	26.37	neg	neg	neg	26.55	neg
8	1	inoculated	M4-8	33.97	neg	neg	neg	neg	neg
9	1	inoculated	M4-9	28.97	neg	neg	neg	28.83	neg
10	1	inoculated	M4-10	29.52	neg	neg	neg	30.52	neg
11	1	sentinel	M4-11	neg	neg	neg	neg	neg	neg
12	1	sentinel	M4-12	neg	neg	neg	neg	neg	neg
13	1	sentinel	M4-13	neg	neg	neg	neg	neg	neg
14	1	sentinel	M4-14	neg	neg	neg	neg	neg	neg
15	1	inoculated	M4-1	neg	neg	neg	neg	neg	neg
16	1	inoculated	M4-2	25.93	neg	neg	neg	27.27	neg
17	1	inoculated	M4-3	32.70	neg	neg	neg	38.80	neg
18	1	inoculated	M4-4	neg	neg	neg	neg	neg	neg
19	1	inoculated	M4-5	neg	neg	neg	neg	neg	neg
20	1	inoculated	M4-6	neg	37.46	neg	neg	neg	neg
21	1	inoculated	M4-7	28.99	35.40	neg	neg	31.12	neg
22	1	inoculated	M4-8	34.07	neg	neg	neg	neg	neg
23	1	inoculated	M4-9	29.36	27.60	neg	neg	33.10	31.52
24	1	inoculated	M4-10	27.68	33.17	neg	neg	28.87	neg
25	1	sentinel	M4-11	neg	neg	neg	neg	neg	neg
26	1	sentinel	M4-12	neg	neg	neg	neg	neg	neg
27	1	sentinel	M4-13	neg	neg	neg	neg	neg	neg
28	1	sentinel	M4-14	neg	neg	neg	neg	neg	neg
29	4	inoculated	M4-1	36.16	neg	neg	neg	neg	neg
30	4	inoculated	M4-3	30.85	29.53	neg	neg	32.79	29.53
31	4	inoculated	M4-4	34.64	40.45	neg	neg	neg	neg
32	4	inoculated	M4-5	36.20	neg	neg	neg	neg	neg
33	4	inoculated	M4-6	34.08	32.42	neg	neg	neg	neg
34	4	inoculated	M4-8	30.42	25.98	neg	neg	32.81	26.62
35	4	inoculated	M4-9	31.90	27.92	neg	neg	neg	29.07
36	4	inoculated	M4-10	22.51	25.98	neg	neg	22.21	25.75
37	4	sentinel	M4-11	37.54	33.11	neg	neg	neg	33.54
38	4	sentinel	M4-12	37.12	32.63	neg	neg	neg	32.67
39	6	inoculated	M4-1	35.02	neg	neg	neg	neg	neg
40	6	inoculated	M4-3	26.04	29.14	neg	neg	26.71	30.98
41	6	inoculated	M4-4	32.80	33.81	neg	neg	neg	neg
42	6	inoculated	M4-5	37.29	35.20	neg	neg	neg	neg
43	6	sentinel	M4-11	24.68	31.12	neg	neg	24.64	26.12
44	6	sentinel	M4-12	32.47	32.27	neg	neg	neg	27.59
45	7	sentinel	M4-12	28.19	25.45	neg	neg	28.89	25.85
46	8	inoculated	M4-3	35.00	30.86	neg	neg	neg	24.03
47	8	inoculated	M4-4	23.21	22.80	neg	neg	23.83	neg
48	9	inoculated	M4-1	neg	24.91	neg	neg	neg	26.32
49	9	inoculated	M4-5	neg	30.10	neg	neg	neg	32.11
50	10	inoculated	M4-5	30.06	27.60	neg	neg	29.43	28.85

group M5				Real-time RT-PCR					
	dpi	status	animal ID	M1.2 OP	M1.2 CL	H7 LP OP	H7 LP CL	H7 HP OP	H7 HP CL
1	1	inoculated	M5-1	neg	neg	neg	neg	neg	neg
2	1	inoculated	M5-2	neg	neg	neg	neg	neg	neg
3	1	inoculated	M5-3	38.97	neg	neg	neg	neg	neg
4	1	inoculated	M5-4	neg	neg	neg	neg	neg	neg
5	1	inoculated	M5-5	31.43	neg	neg	neg	32.30	neg
6	1	inoculated	M5-6	35.49	neg	neg	neg	neg	neg
7	1	inoculated	M5-7	neg	neg	neg	neg	neg	neg
8	1	inoculated	M5-8	neg	neg	neg	neg	neg	neg
9	1	inoculated	M5-9	neg	neg	neg	neg	neg	neg
10	1	inoculated	M5-10	28.39	neg	neg	neg	29.17	neg
11	1	sentinel	M5-11	neg	neg	neg	neg	neg	neg
12	1	sentinel	M5-12	neg	neg	neg	neg	neg	neg
13	1	sentinel	M5-13	33.29	neg	neg	neg	31.22	neg
14	1	sentinel	M5-14	neg	neg	neg	neg	neg	neg
15	2	inoculated	M5-1	31.86	37.46	neg	neg	35.73	neg
16	2	inoculated	M5-2	neg	neg	neg	neg	neg	neg
17	2	inoculated	M5-3	32.68	neg	neg	neg	36.79	neg
18	2	inoculated	M5-4	neg	neg	neg	neg	neg	neg
19	2	inoculated	M5-5	31.73	25.57	neg	neg	37.93	26.70
20	2	inoculated	M5-6	38.47	38.30	neg	neg	neg	neg
21	2	inoculated	M5-7	neg	39.86	neg	neg	neg	neg
22	2	inoculated	M5-8	neg	neg	neg	neg	neg	neg
23	2	inoculated	M5-9	39.07	neg	neg	neg	28.62	neg
24	2	inoculated	M5-10	26.53	36.27	neg	neg	neg	neg
25	2	sentinel	M5-11	33.82	28.18	neg	neg	neg	32.59
26	2	sentinel	M5-12	neg	neg	neg	neg	neg	neg
27	2	sentinel	M5-13	32.04	36.18	neg	neg	34.27	neg
28	2	sentinel	M5-14	34.74	neg	neg	neg	neg	neg
29	4	inoculated	M5-1	31.17	33.07	neg	neg	30.59	neg
30	4	inoculated	M5-2	33.68	27.44	neg	neg	neg	28.28
31	4	inoculated	M5-3	27.64	30.64	neg	neg	26.86	32.14
32	4	inoculated	M5-4	neg	neg	neg	neg	neg	neg
33	4	inoculated	M5-5	25.46	27.13	neg	neg	25.66	26.23
34	4	inoculated	M5-7	30.62	28.00	neg	neg	31.63	28.23
35	4	inoculated	M5-8	32.98	34.17	neg	neg	neg	neg
36	4	sentinel	M5-9	31.56	33.39	neg	neg	31.77	neg
37	4	sentinel	M5-11	27.29	27.15	neg	neg	26.66	28.36
38	4	sentinel	M5-13	26.08	25.32	neg	neg	26.15	25.27
39	5	inoculated	M5-1	27.08	28.86	neg	neg	28.48	26.73
40	5	inoculated	M5-3	26.52	24.55	neg	neg	26.08	28.36
41	5	sentinel	M5-11	28.00	neg	neg	neg	24.55	23.44
42	6	inoculated	M5-2	28.39	27.50	neg	neg	27.60	27.16
43	6	inoculated	M5-4	25.87	26.82	neg	neg	26.36	27.15
44	6	inoculated	M5-7	27.20	27.73	neg	neg	26.40	26.41
45	6	inoculated	M5-8	28.02	27.52	neg	neg	27.71	26.33
46	6	inoculated	M5-9	27.69	29.19	neg	neg	27.27	28.79
47	7	inoculated	M5-7	19.55	26.10	neg	neg	19.14	25.64
48	7	sentinel	M5-14	25.64	22.45	neg	neg	25.30	22.14

group M5.7				Real-time RT-PCR					
	dpi	status	animal ID	M1.2 OP	M1.2 CL	H7 LP OP	H7 LP CL	H7 HP OP	H7 HP CL
1	1	inoculated	M5.7-1	neg	neg	neg	neg	neg	neg
2	1	inoculated	M5.7-2	23.01	neg	neg	neg	25.27	neg
3	1	inoculated	M5.7-3	33.19	neg	neg	neg	neg	neg
4	1	inoculated	M5.7-4	23.87	35.23	neg	neg	27.07	neg
5	1	inoculated	M5.7-5	25.00	neg	neg	neg	27.49	neg
6	1	inoculated	M5.7-6	25.43	38.44	neg	neg	27.30	neg
7	1	inoculated	M5.7-7	24.65	38.05	neg	neg	27.06	neg
8	1	inoculated	M5.7-8	26.57	neg	neg	neg	28.58	neg
9	1	inoculated	M5.7-9	21.50	36.21	neg	neg	22.79	neg
10	1	inoculated	M5.7-10	22.69	33.09	neg	neg	23.90	neg
11	1	sentinel	M5.7-11	neg	neg	neg	neg	neg	neg
12	1	sentinel	M5.7-12	36.30	36.57	neg	neg	neg	neg
13	1	sentinel	M5.7-13	neg	neg	neg	neg	neg	neg
14	1	sentinel	M5.7-14	neg	neg	neg	neg	neg	neg
15	2	inoculated	M5.7-1	38.65	neg	neg	neg	neg	neg
16	2	inoculated	M5.7-2	29.51	24.67	neg	neg	30.74	26.43
17	2	inoculated	M5.7-3	38.13	25.03	neg	neg	neg	neg
18	2	inoculated	M5.7-4	30.83	24.15	neg	neg	36.54	neg
19	2	inoculated	M5.7-5	29.42	35.06	neg	neg	29.97	25.59
20	2	inoculated	M5.7-6	26.64	24.47	neg	neg	27.01	25.22
21	2	inoculated	M5.7-7	30.86	27.18	neg	neg	33.94	neg
22	2	inoculated	M5.7-8	28.07	35.30	neg	neg	29.72	26.10
23	2	inoculated	M5.7-9	31.02	35.63	neg	neg	37.01	30.20
24	2	inoculated	M5.7-10	29.44	31.76	neg	neg	neg	neg
25	2	sentinel	M5.7-11	neg	37.42	neg	neg	neg	neg
26	2	sentinel	M5.7-12	neg	neg	neg	neg	neg	neg
27	2	sentinel	M5.7-13	36.71	neg	neg	neg	neg	neg
28	2	sentinel	M5.7-14	38.17	neg	neg	neg	33.96	neg
29	3	inoculated	M5.7-7	26.59	25.05	neg	neg	27.77	25.37
30	3	inoculated	M5.7-9	25.15	25.40	neg	neg	26.55	26.28
31	3	inoculated	M5.7-10	26.10	29.20	neg	neg	27.27	30.08
32	4	inoculated	M5.7-1	26.36	24.17	neg	27.96	32.17	24.44
33	4	inoculated	M5.7-4	28.50	28.20	neg	neg	26.20	25.06
34	4	inoculated	M5.7-5	23.37	22.88	neg	neg	28.25	28.46
35	4	inoculated	M5.7-6	27.58	neg	neg	neg	33.22	24.06
36	4	inoculated	M5.7-7	neg	neg	neg	neg	neg	neg
37	4	inoculated	M5.7-8	21.08	26.31	neg	neg	neg	26.28
38	4	inoculated	M5.7-9	neg	neg	neg	neg	21.00	27.05
39	4	sentinel	M5.7-12	neg	39.62	neg	neg	neg	neg
40	4	sentinel	M5.7-13	neg	37.15	neg	neg	neg	neg
41	5	inoculated	M5.7-4	24.26	28.06	neg	neg	26.84	26.48
42	5	inoculated	M5.7-6	17.33	24.50	neg	neg	16.25	23.57
43	6	inoculated	M5.7-1	26.54	23.44	neg	neg	25.41	22.13
44	6	sentinel	M5.7-12	31.08	32.77	neg	neg	32.87	neg
45	6	sentinel	M5.7-13	36.01	36.41	neg	neg	neg	neg
46	8	sentinel	M5.7-12	27.77	27.45	neg	neg	27.37	27.31
47	9	sentinel	M5.7-13	24.63	34.20	neg	neg	23.47	neg

group M6				Real-time RT-PCR					
	dpi	status	animal ID	M1.2 OP	M1.2 CL	H7 LP OP	H7 LP CL	H7 HP OP	H7 HP CL
1	1	inoculated	M6-1	32.49	neg	neg	neg	neg	neg
2	1	inoculated	M6-2	31.82	neg	neg	neg	34.12	24.45
3	1	inoculated	M6-3	38.26	neg	neg	neg	neg	34.34
4	1	inoculated	M6-4	neg	neg	neg	neg	neg	neg
5	1	inoculated	M6-5	31.54	neg	neg	neg	32.88	neg
6	1	inoculated	M6-6	37.54	neg	neg	neg	neg	neg
7	1	inoculated	M6-7	24.11	neg	neg	neg	24.10	neg
8	1	inoculated	M6-8	30.29	neg	neg	neg	31.22	neg
9	1	inoculated	M6-9	22.13	37.44	neg	neg	neg	neg
10	1	inoculated	M6-10	33.41	neg	neg	neg	neg	neg
11	1	sentinel	M6-11	neg	neg	neg	neg	neg	neg
12	1	sentinel	M6-12	neg	neg	neg	neg	neg	neg
13	1	sentinel	M6-13	neg	neg	neg	neg	neg	neg
14	1	sentinel	M6-14	neg	neg	neg	neg	neg	neg
15	2	inoculated	M6-1	35.28	35.60	neg	neg	neg	neg
16	2	inoculated	M6-2	31.63	36.07	neg	neg	35.08	neg
17	2	inoculated	M6-3	36.01	39.55	neg	neg	neg	neg
18	2	inoculated	M6-4	31.10	35.87	neg	neg	32.45	neg
19	2	inoculated	M6-5	34.89	36.06	neg	neg	neg	neg
20	2	inoculated	M6-6	31.68	29.47	neg	neg	34.42	34.16
21	2	inoculated	M6-7	23.07	29.20	neg	neg	23.26	35.23
22	2	inoculated	M6-8	35.71	neg	neg	neg	neg	neg
23	2	inoculated	M6-9	28.54	26.72	neg	neg	29.72	27.05
24	2	inoculated	M6-10	31.54	35.22	neg	neg	32.02	neg
25	2	sentinel	M6-11	neg	neg	neg	neg	neg	neg
26	2	sentinel	M6-12	neg	neg	neg	neg	neg	neg
27	2	sentinel	M6-13	neg	neg	neg	neg	neg	neg
28	2	sentinel	M6-14	neg	neg	neg	neg	neg	neg
29	3	inoculated	M6-4	27.11	28.04	neg	neg	28.54	28.98
30	3	inoculated	M6-5	28.87	31.31	neg	neg	30.04	32.90
31	3	inoculated	M6-6	28.47	30.49	neg	neg	30.04	33.28
32	4	inoculated	M6-1	29.22	27.83	neg	neg	30.14	30.73
33	4	inoculated	M6-2	26.82	23.61	neg	neg	26.66	25.15
34	4	inoculated	M6-3	29.60	30.52	neg	neg	29.59	neg
35	4	inoculated	M6-7	25.01	35.29	neg	neg	25.62	neg
36	4	inoculated	M6-10	27.71	27.06	neg	neg	28.54	29.24
37	4	sentinel	M6-12	29.37	27.62	neg	neg	30.21	28.54
38	4	sentinel	M6-14	34.01	33.25	neg	neg	neg	neg
39	5	inoculated	M6-2	26.21	26.11	neg	neg	25.04	25.08
40	5	inoculated	M6-7	25.00	25.26	neg	neg	23.35	24.68
41	6	inoculated	M6-3	25.05	25.45	neg	neg	24.74	24.29
42	6	inoculated	M6-10	22.06	28.53	neg	neg	20.92	26.55
43	6	sentinel	M6-12	32.83	34.68	neg	neg	35.27	neg
44	6	sentinel	M6-14	32.69	28.30	neg	neg	neg	27.45

group B				Real-time RT-PCR					
	dpi	status	animal ID	M1.2 OP	M1.2 CL	H7 LP OP	H7 LP CL	H7 HP OP	H7 HP CL
1	1	inoculated	B-1	30.29	neg	34.61	neg	neg	neg
2	1	inoculated	B-2	25.81	38.69	29.43	neg	neg	neg
3	1	inoculated	B-3	22.24	36.74	26.40	neg	neg	neg
4	1	inoculated	B-4	23.24	38.38	26.30	neg	neg	neg
5	1	inoculated	B-5	25.97	neg	29.14	neg	neg	neg
6	1	inoculated	B-6	25.68	36.66	29.37	neg	neg	neg
7	1	inoculated	B-7	22.44	neg	27.66	neg	neg	neg
8	1	inoculated	B-8	32.16	neg	36.07	neg	neg	neg
9	1	inoculated	B-9	27.61	neg	31.17	neg	neg	neg
10	1	inoculated	B-10	25.59	neg	30.63	neg	neg	neg
11	1	sentinel	B-11	neg	neg	neg	neg	neg	neg
12	1	sentinel	B-12	neg	neg	neg	neg	neg	neg
13	1	sentinel	B-13	neg	neg	neg	neg	neg	neg
14	1	sentinel	B-14	neg	neg	neg	neg	neg	neg
15	2	inoculated	B-1	neg	neg	neg	neg	neg	neg
16	2	inoculated	B-2	37.42	31.98	33.80	neg	neg	neg
17	2	inoculated	B-3	neg	38.04	neg	neg	neg	neg
18	2	inoculated	B-4	35.38	35.24	neg	neg	neg	neg
19	2	inoculated	B-5	27.12	neg	30.64	neg	neg	neg
20	2	inoculated	B-6	30.09	38.42	37.29	34.56	neg	neg
21	2	inoculated	B-7	35.09	neg	neg	neg	neg	neg
22	2	inoculated	B-8	30.45	neg	neg	neg	neg	neg
23	2	inoculated	B-9	29.72	neg	36.43	neg	neg	neg
24	2	inoculated	B-10	26.29	21.09	32.91	neg	neg	neg
25	2	sentinel	B-11	31.72	28.05	neg	neg	neg	neg
26	2	sentinel	B-12	38.20	neg	neg	neg	neg	neg
27	2	sentinel	B-13	neg	neg	neg	neg	neg	neg
28	2	sentinel	B-14	neg	38.13	neg	neg	neg	neg
29	4	inoculated	B-2	26.98	36.86	30.41	neg	neg	neg
30	4	inoculated	B-3	29.08	37.00	30.88	neg	neg	neg
31	4	inoculated	B-4	27.67	24.65	31.41	30.47	neg	neg
32	4	inoculated	B-5	27.45	32.79	32.30	32.68	neg	neg
33	4	inoculated	B-6	26.44	24.38	33.11	29.05	neg	neg
34	4	inoculated	B-7	30.28	24.92	31.03	29.52	neg	neg
35	4	inoculated	B-8	27.70	neg	34.64	neg	neg	neg
36	4	inoculated	B-10	neg	29.06	33.32	32.07	neg	neg
37	4	sentinel	B-12	36.22	22.61	33.59	26.42	neg	neg
38	4	sentinel	B-13	37.27	30.83	neg	neg	neg	neg
39	6	inoculated	B-2	neg	33.80	neg	neg	neg	neg
40	6	inoculated	B-3	34.95	neg	neg	neg	neg	neg
41	6	inoculated	B-4	35.87	30.08	neg	35.61	neg	neg
42	6	inoculated	B-5	30.42	35.42	29.07	neg	neg	neg
43	6	inoculated	B-6	31.55	33.76	31.58	neg	neg	neg
44	6	inoculated	B-7	32.24	32.57	34.91	neg	neg	neg
45	6	inoculated	B-8	neg	36.46	neg	neg	neg	neg
46	6	inoculated	B-10	35.15	neg	34.19	neg	neg	neg
47	6	sentinel	B-12	35.34	37.45	neg	neg	neg	neg
48	6	sentinel	B-13	38.49	33.24	neg	neg	neg	neg
49	9	inoculated	B-2	neg	neg	neg	neg	neg	neg
50	9	inoculated	B-3	neg	neg	neg	neg	neg	neg
51	9	inoculated	B-4	neg	33.58	neg	neg	neg	neg
52	9	inoculated	B-5	37.96	31.35	neg	neg	neg	neg
53	9	inoculated	B-6	neg	31.64	neg	neg	neg	neg
54	9	inoculated	B-7	40.90	30.61	neg	37.34	neg	neg
55	9	inoculated	B-8	neg	neg	neg	neg	neg	neg
56	9	inoculated	B-10	neg	neg	neg	neg	neg	neg
57	9	sentinel	B-12	neg	neg	neg	neg	neg	neg
58	9	sentinel	B-13	neg	37.80	neg	neg	neg	neg
59	13	inoculated	B-2	neg	38.10	neg	neg	neg	neg
60	13	inoculated	B-3	neg	37.02	neg	neg	neg	neg
61	13	inoculated	B-4	neg	32.84	neg	36.92	neg	neg
62	13	inoculated	B-5	29.59	38.32	31.59	neg	neg	neg
63	13	inoculated	B-6	neg	34.26	neg	38.03	neg	neg
64	13	inoculated	B-7	neg	neg	neg	neg	neg	neg
65	13	inoculated	B-8	neg	38.85	neg	neg	neg	neg
66	13	inoculated	B-10	neg	neg	neg	neg	neg	neg
67	13	sentinel	B-12	neg	35.23	neg	neg	neg	neg
68	13	sentinel	B-13	neg	25.30	neg	31.71	neg	neg

Supplemental Table 3a. Area-under-curve (AUC) values from viral shedding analyses of the inoculated chickens (supplement to Figure 1c).

		AUC values	
Group	**Shedding***	**Co-infection group**	**Mono-infection group**
C1 & M1	OP	0	0
C3 & M3	OP	0	35.206
C4 & M4	OP	46.7115	40.1555
C5 & M5	OP	36.48566667	48.637375
C5.7 & M5.7	OP	48.68225	58.0387619
B & M6	OP	0	53.929
C1 & M1	CL	0	0
C3 & M3	CL	0	46.4385
C4 & M4	CL	41.435	46.377
C5 & M5	CL	25.95425	43.797
C5.7 & M5.7	CL	35.434125	55.40552381
B & M6	CL	0	41.0825

*OP = oropharyngeal swab; *CL = cloacal swab

Supplemental Table 3b. P-values of viral shedding analyses (supplement to Figure 1c).

Group	**CL***	**OP***
C3 vs. M3	0.02913886	0.06293646
C4 vs. M4	0.16270325	0.14744695
C5 vs. M5	0.58302491	0.79220779
C5.7 vs. M5.7	0.83066424	0.76190476
B vs. M6	0.00277843	0.00277843

*OP = oropharyngeal swab; *CL = cloacal swab

Supplemental Table 4a. Mean death time calculations of 10-and 14-day old embryonated chicken eggs (supplement to Figure 3a).

Group	Embryo	MDT (hpi)	
		10-day-old ECE	14-day-old ECE
C1	C1-1	72	72
	C1-2	72	72
	C1-3	72	120
	C1-4	72	96
	C1-5	72	120
M1	M1-1	120	120
	M1-2	120	120
	M1-3	120	120
	M1-4	120	120
	M1-5	120	120
C3	C3-1	48	96
	C3-2	72	48
	C3-3	72	72
	C3-4	48	96
	C3-5	48	72
M3	M3-1	48	48
	M3-2	42	48
	M3-3	48	48
	M3-4	48	48
	M3-5	48	48
C4	C4-1	48	76
	C4-2	72	76
	C4-3	72	72
	C4-4	72	72
	C4-5	48	72
M4	M4-1	42	42
	M4-2	24	42
	M4-3	42	42
	M4-4	42	42
	M4-5	42	42
C5	C5-1	52	76
	C5-2	52	76
	C5-3	48	76
	C5-4	48	24
	C5-5	48	48
M5	M5-1	28	42
	M5-2	28	42
	M5-3	42	42
	M5-4	42	42
	M5-5	28	42
C5.7	C5.7-1	48	48
	C5.7-2	48	48
	C5.7-3	24	76
	C5.7-4	48	76
	C5.7-5	48	48
M5.7	M5.7-1	28	42
	M5.7-2	28	42
	M5.7-3	28	42
	M5.7-4	42	24
	M5.7-5	42	42
B	B-1	96	96
	B-2	96	120
	B-3	48	76
	B-4	120	120
	B-5	120	76
M6	M6-1	24	24
	M6-2	28	42
	M6-3	28	24
	M6-4	24	24
	M6-5	42	42

Supplemental Table 4b. P-values of *in ovo* experiments: (A) comparison of MDTs between co- and mono-infection groups in 10- and 14-day old embryonated chicken eggs (Mantel-Haenszel-logrank test) and (B) comparison of MDTs of each of the mono- and co-infection groups within 10- and 14-day old ECEs (supplement to Figure 3a).

(A)

	ECEs	
Group	**10-day old**	**14-day old**
C1 vs. M1	9.63E-07	0.023185644
C3 vs. M3	0.01332194	4.94E-05
C4 vs. M4	9.34E-06	3.41E-07
C5 vs. M5	1.60E-05	0.000315491
C5.7 vs. M5.7	0.00402611	9.34E-06
B vs. M6	1.60E-09	4.63E-09

(B)

Group	**10- vs. 14-day old ECEs**
C1	0.00134865
C3	0.010914202
C4	0.016791821
C5	0.014158931
C5.7	0.006742087
M1	1
M3	0.291840545
M4	0.28274546
M5	0.026888454
M5.7	0.235044451
M6	0.67087816
B	0.919851039

Supplemental Table 5. RT-qPCR results of harvested amnio-allantoic fluids of 10-and 14-day old embryonated chicken eggs infected with LP and/or HPAIV (*in ovo* experiment) (supplement to Figure 3b).

Egg ID	10-day-old ECE			14-day-old ECE		
	M1.2	H7 LP	H7 HP	M1.2	H7 LP	H7 HP
C1-1	14.83	20.14	32.32	17.69	21.17	neg
C1-2	13.47	18.97	neg	17.01	20.16	neg
C1-3	15.39	20.02	neg	17.09	20.47	neg
C1-4	13.77	19.66	neg	16.39	19.36	neg
C1-5	18.31	23.2	neg	18.95	22.09	neg
C3-1	13.87	20.04	neg	17.51	21.63	neg
C3-2	14.05	19.41	25.22	neg	20.51	neg
C3-3	14.75	20.02	neg	neg	21.66	neg
C3-4	21.08	26.37	neg	15.86	19.66	neg
C3-5	16.64	19.03	neg	18.07	22.29	neg
C4-1	13.99	19.51	neg	15.06	17.84	34.07
C4-2	13.1	18.77	neg	14.4	17.26	31.23
C4-3	13.15	19.18	26.91	17.01	19.7	neg
C4-4	13.23	18.36	neg	15.64	18.35	neg
C4-5	13.48	18.48	neg	15.21	18.12	neg
C5-1	13.6	19.49	neg	17.99	21.23	24.76
C5-2	13.11	18.3	neg	16.78	19.68	28.83
C5-3	14.4	20.04	neg	21.98	25.95	neg
C5-4	13.2	18.49	neg	18.05	22.52	35.52
C5-5	13.11	18.33	31.45	13.96	17.42	neg
C5.7-1	13.82	19.53	neg	15.26	18.69	25.58
C5.7-2	14.32	19.67	32.5	18.17	20.89	34.47
C5.7-3	13.93	19.32	neg	18.12	22.05	29.19
C5.7-4	13.32	19.4	34.29	18.74	23.46	23.7
C5.7-5	34.23	neg	neg	17.66	21.9	34.18
M1-1	14.78	neg	19.55	14.56	neg	20.13
M1-2	13.86	neg	18.21	14.67	neg	20.23
M1-3	13.24	neg	17.58	13.64	neg	18.72
M1-4	13.63	neg	16.65	14.63	neg	19.83
M1-5	14.36	neg	16.77	14.38	neg	20.18
M3-1	14.64	neg	17.69	14.04	neg	19.79
M3-2	13.12	neg	17.47	14.61	neg	20.29
M3-3	13.91	neg	18.25	13.97	neg	19.25
M3-4	13.24	neg	18.87	13.44	neg	19.02
M3-5	13.33	neg	17.58	15.15	neg	19.64
M4-1	16.639	neg	19.75	15.63	neg	20.53
M4-2	13.98	neg	19.2	13.94	neg	19.2
M4-3	14.97	neg	20.82	14.54	neg	20.82
M4-4	15.98	neg	17.72	16.21	neg	17.72
M4-5	15.61	neg	15.54	14.36	neg	15.54
M5-1	13.64	neg	15.11	14.64	neg	16.57
M5-2	13.86	neg	15.78	15.8	neg	17.99
M5-3	14.18	neg	15.58	16.06	neg	17.89
M5-4	13.99	neg	14.68	13.76	neg	15.68
M5-5	14.36	neg	16.74	15.52	neg	17.46
M5.7-1	15.72	neg	17.56	16.92	neg	18.4
M5.7-2	14.33	neg	15.26	15.16	neg	16.86
M5.7-3	15.69	neg	16.11	15.28	neg	16.66
M5.7-4	14.25	neg	15.09	15.01	neg	17.06
M5.7-5	14.39	neg	16.63	15.48	neg	17.53
M6-1	14.83	neg	15.15	14.53	neg	16.47
M6-2	15.88	neg	21.26	18.07	neg	19.26
M6-3	15.97	neg	18.67	16.29	neg	18.37
M6-4	14.11	neg	15.45	14.71	neg	16.62
M6-5	14.21	neg	16.52	15.29	neg	17.78
B-1	15.82	19.02	neg	18.15	21.13	neg
B-2	14.28	18.08	neg	17.58	21.21	neg
B-3	15.43	18.51	neg	17.75	21.12	neg
B-4	15.07	18.38	neg	17.65	21.41	neg
B-5	19.6	23.29	neg	14.54	18.09	neg

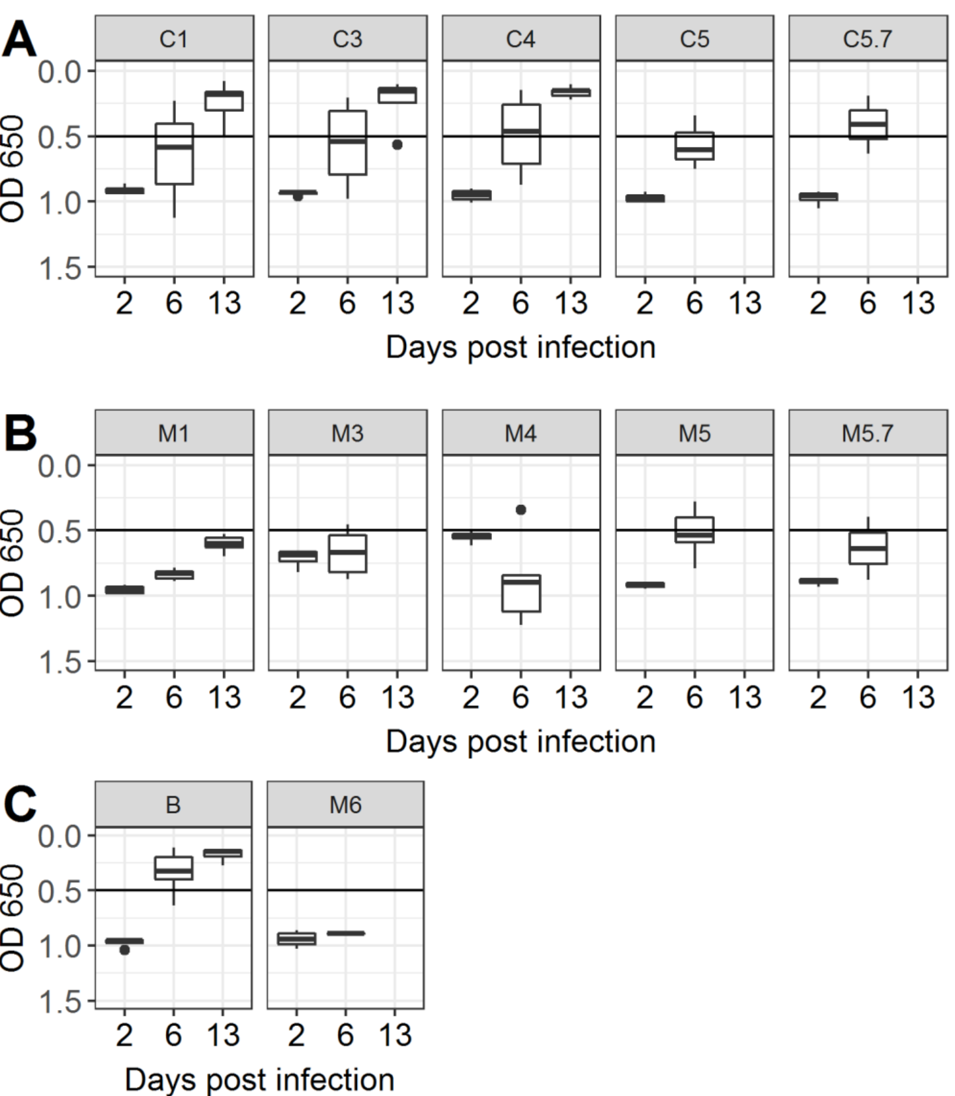
Supplemental Table 6. RT-qPCR results of tissues selected from of 10-and 14-day old embryonated chicken eggs infected with LP/HPAIV (*in ovo* experiment; co-infections).

10-day-old ECE

		heart			brain			liver		
Group	**embryo ID**	**M1.2**	**H7 LP**	**H7 HP**	**M1.2**	**H7 LP**	**H7 HP**	**M1.2**	**H7 LP**	**H7 HP**
C1	C1-1	22.16	neg	neg	20.08	neg	27.89	20.38	26.84	neg
	C1-2	20.37	neg	neg	22.13	neg	25.35	20.78	26.74	neg
	C1-3	20.34	neg	neg	20.56	neg	27.31	20.63	neg	neg
C3	C3-1	15.79	20.4	19.12	15.5	20.66	21.08	15.82	23.65	18.85
	C3-2	13.93	25.45	16.9	13.13	19.38	17.53	15.11	neg	19.34
	C3-3	14.71	neg	17.24	15.25	neg	18.11	16.4	neg	19.38
C4	C4-1	17.87	25.59	22.11	16.74	23.98	28.78	19.12	25.42	27.74
	C4-2	13.87	neg	16.86	13.75	neg	17.23	16.44	34	20.55
	C4-3	13.27	neg	15.55	14.8	25.75	18.2	15.87	21.62	19.55
C5	C5-1	15.14	neg	18.42	14.24	neg	18.96	13.94	neg	17
	C5-2	15.17	neg	16.6	14.69	neg	17.07	16.06	neg	18.45
	C5-3	13.95	neg	16.69	14.52	neg	18.13	15.33	neg	18.78
C5.7	C5.7-1	12.79	neg	15	14.84	neg	16.56	13.55	neg	16.16
	C5.7-2	16.75	neg	18.48	18.29	neg	20.09	17.4	neg	20.05
	C5.7-3	13.47	neg	15.55	14.56	neg	17.51	14.47	neg	19.2
B	B-1	neg	neg	neg	neg	neg	neg	neg	neg	neg
	B-2	37.3	neg	neg	neg	neg	neg	neg	neg	neg
	B-3	neg	neg	neg	38.05	neg	neg	neg	neg	neg

14-day-old ECE

		heart			brain			liver		
Group	**embryo ID**	**M1.2**	**H7 LP**	**H7 HP**	**M1.2**	**H7 LP**	**H7 HP**	**M1.2**	**H7 LP**	**H7 HP**
C1	C1-1	33.74	neg	neg	27.21	neg	neg	30.55	neg	neg
	C1-2	27.22	neg	neg	23.03	26.61	neg	26.6	neg	neg
	C1-3	31.75	neg	neg	28.74	neg	neg	30.04	neg	neg
C3	C3-1	31.85	neg	neg	25.27	neg	neg	27.54	neg	36.7
	C3-2	30.9	neg	neg	24.94	neg	neg	25.1	35.69	neg
	C3-3	30.7	neg	neg	25.87	neg	neg	26.23	neg	neg
C4	C4-1	18.13	neg	19.41	17.49	neg	18.42	21.4	neg	24.49
	C4-2	18.54	neg	20	17.74	neg	19.13	20.25	neg	22.55
	C4-3	17.21	neg	18.17	17.45	neg	18.09	19.42	neg	20.61
C5	C5-1	17.63	neg	18.64	18.1	neg	18.85	19.11	neg	19.16
	C5-2	25.12	neg	25.58	23.84	neg	neg	24.2	neg	31.27
	C5-3	25.63	neg	24.4	19.57	neg	21.41	31.1	neg	28.95
C5.7	C5.7-1	18.22	neg	19.36	18.93	neg	19.5	20.22	neg	21.65
	C5.7-2	16.37	neg	17.42	18.15	neg	19.64	19.86	neg	21.16
	C5.7-3	16.31	neg	17.31	19.48	neg	20.46	19.86	neg	19.85
B	B-1	neg	neg	neg	38.38	neg	neg	neg	neg	neg
	B-2	neg	neg	neg	neg	neg	neg	neg	neg	neg
	B-3	neg	neg	neg	neg	neg	neg	neg	neg	neg

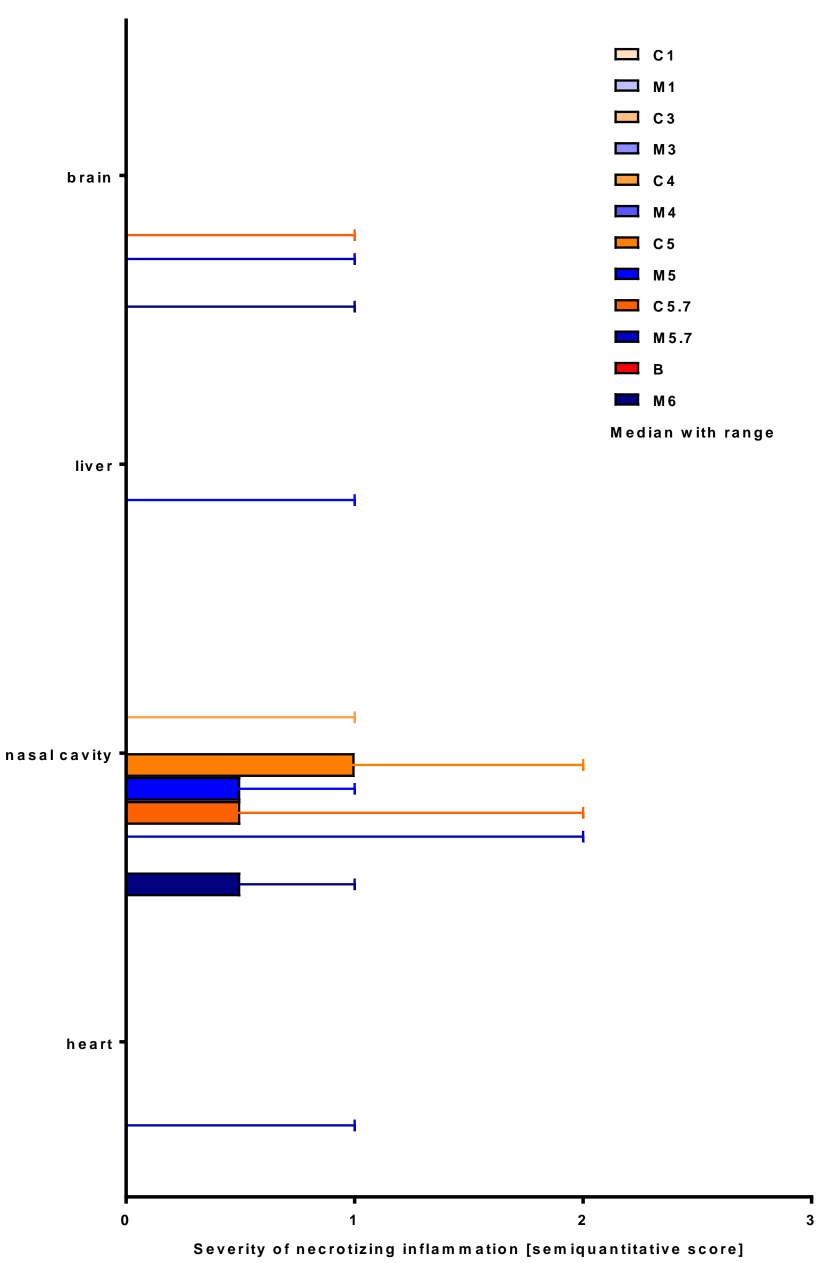


Supplemental Figure 1. Serologic reactions after infection with AIVs of subtypes H7N7 LP and/or HPAIV based on indirect NP-ELISA (OD650. IDEXX) on day 2, 6 and 13 pi. (A) shows co-infection groups C1-C5.7, (B) mono-infection groups M1-M5.7 and (C) control groups B (LP) and M6

Supplemental Figures 2a-d. Histopathological findings and virus tropism as revealed by IHC in inoculated chickens sacrificed at 2 dpi.

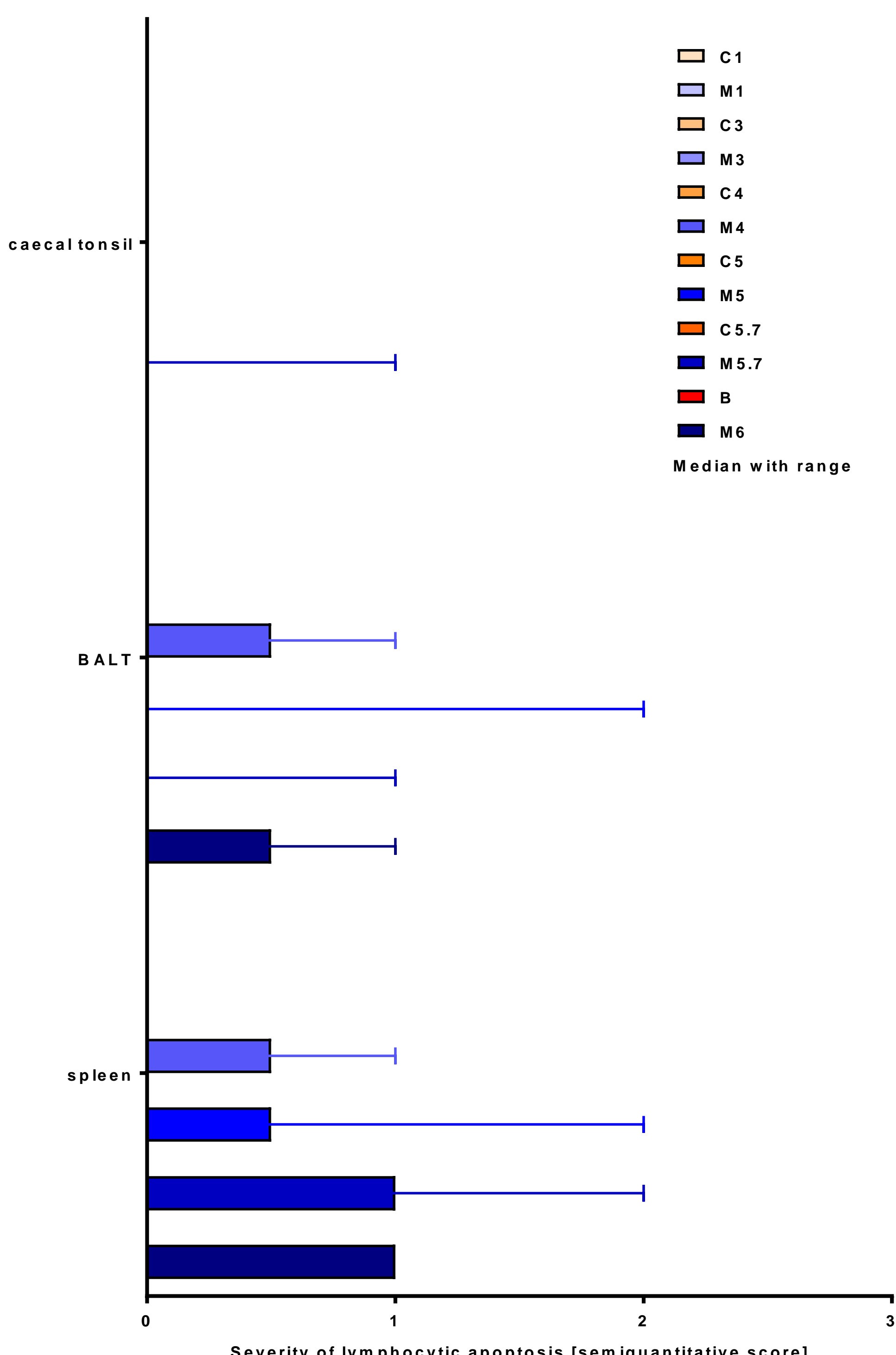
(a) Severitiy of necrotizing inflammation.

0 = negative; 1 = mild; 2 = moderate; 3 = severe.



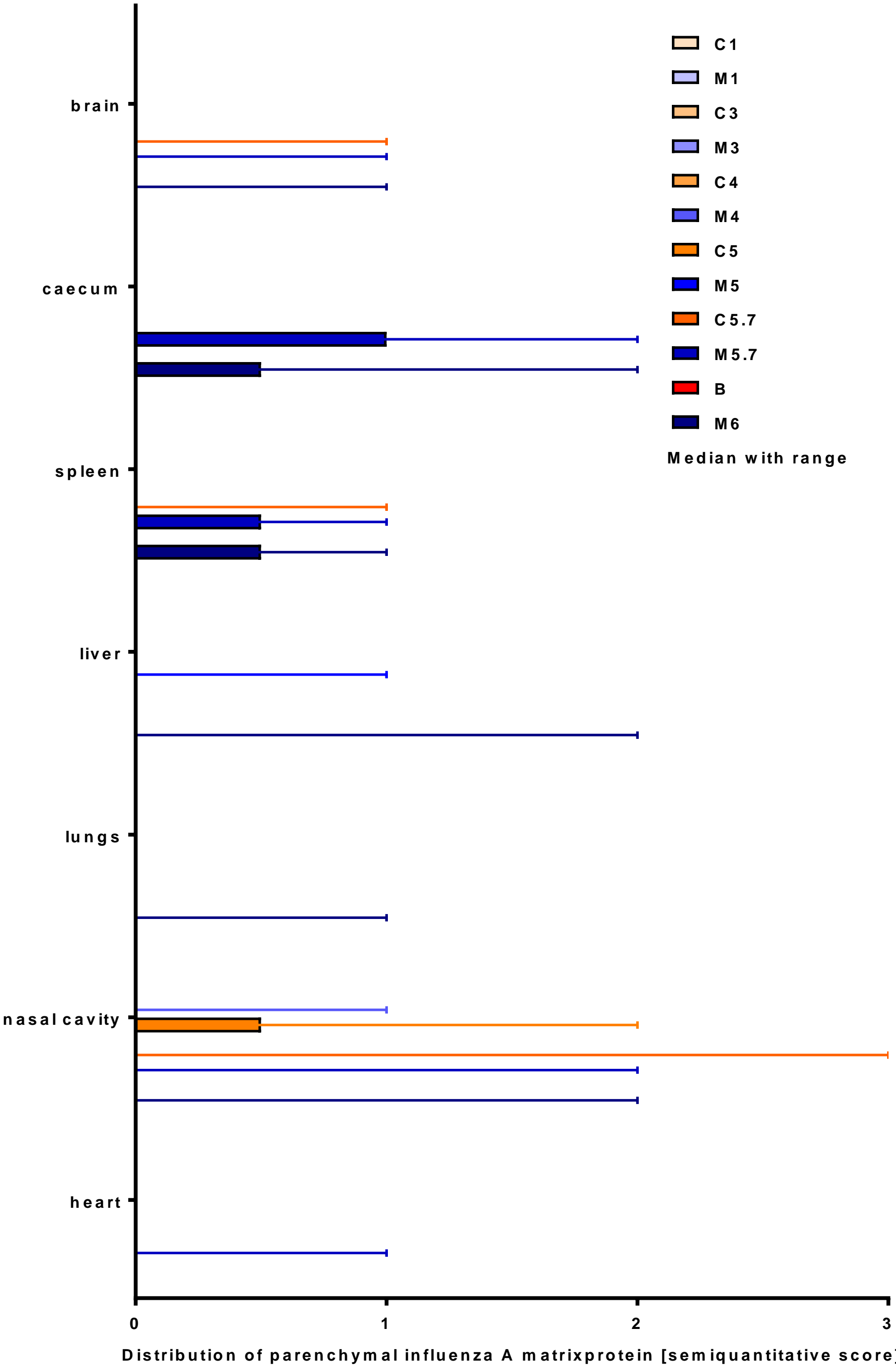
(b) Severity of lymphocytic apoptosis.

0 = negative; 1 = mild; 2 = moderate; 3 = severe.



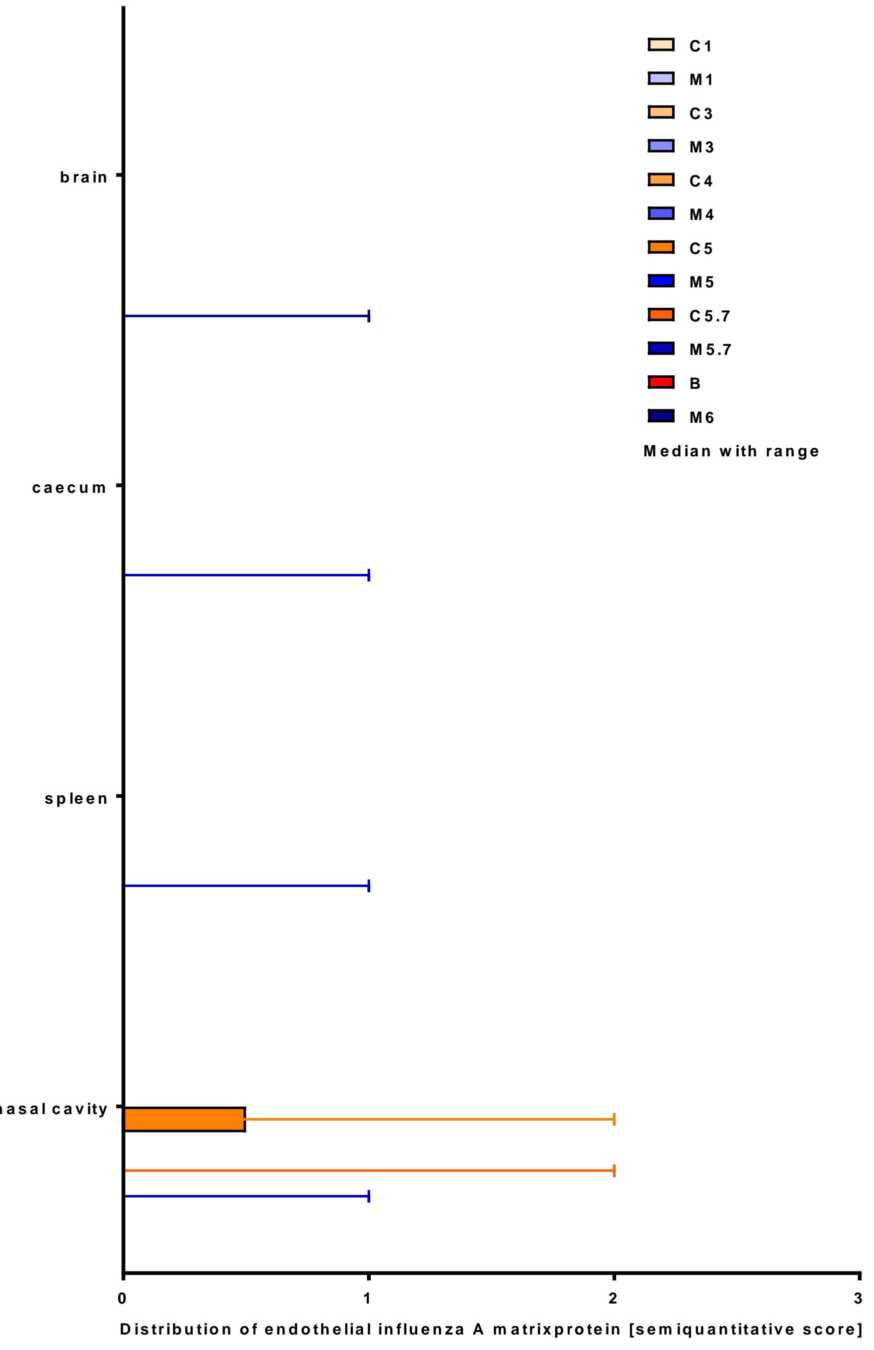
(c) Distribution of parenchymal influenza A matrixprotein.

0 = negative; 1 = focal/oligofocal; 2 = multifocal; 3 = coalesing/diffuse.

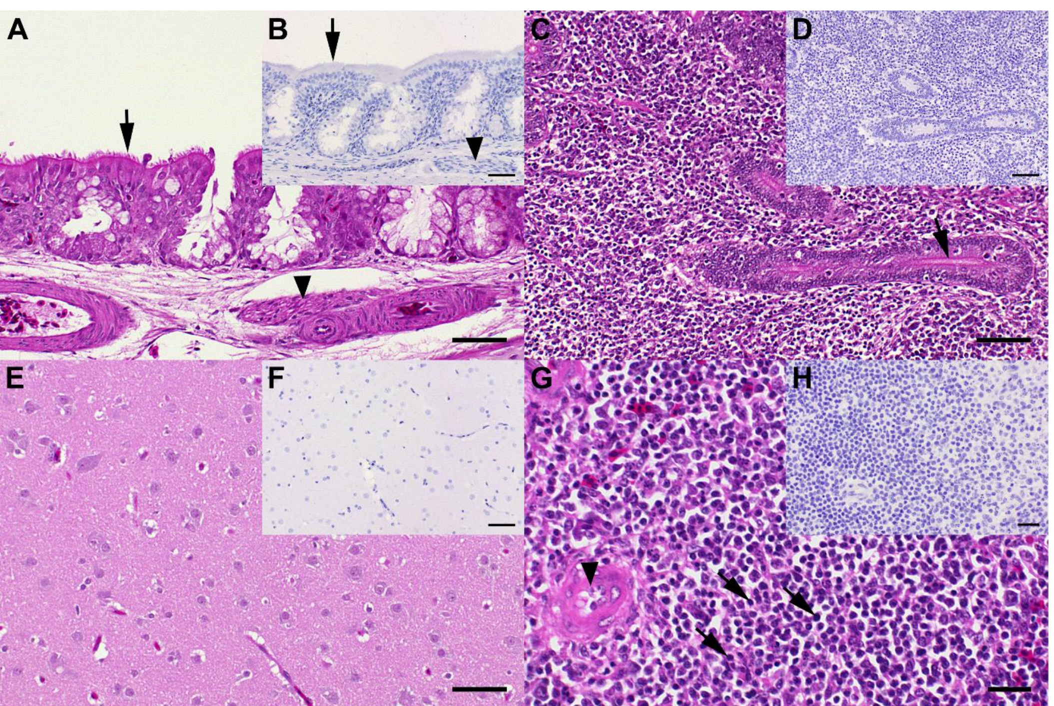


(d) Distribution of endothelial influenza A matrixprotein.

0 = negative; 1 = focal/oligofocal; 2 = multifocal; 3 = coalesing/diffuse.



Supplemental Figure 3. Light microscopy revealed no obvious pathological findings in chicken infected with low pathogenic avian influenza (supplement to Figure 2).



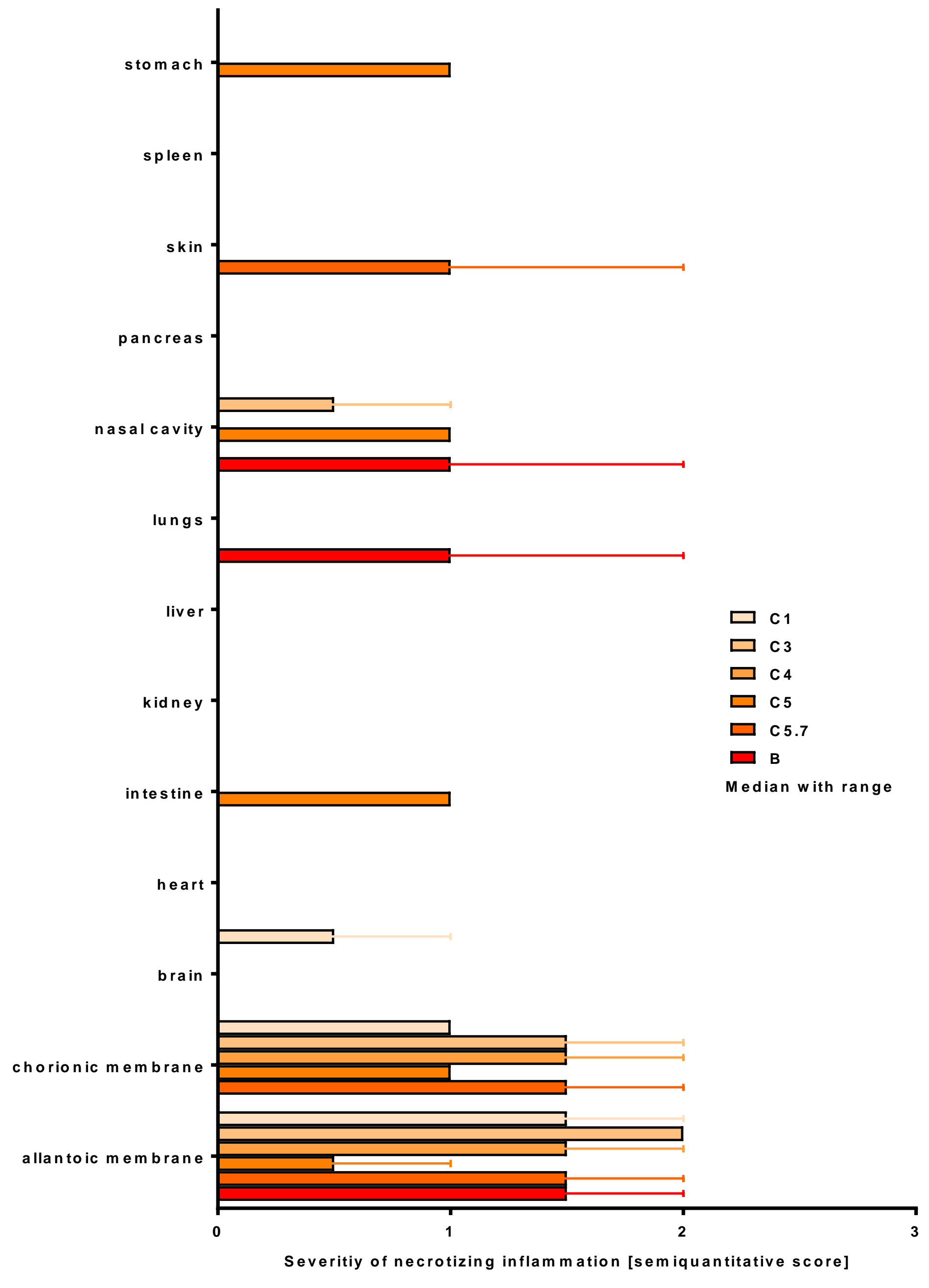
(A) Chicken, P17-884, group B, scheduled euthanasia at 2 dpi, respiratory mucosa. The respiratory mucosa is characterized by a pseudostratified columnar epithelium with prominent apical cilia (arrow) and multifocal intraepithelial mucous glands. The submucosa contains unremarkable blood vessels and nerves (arrowhead). **(B)** Chicken, P17-884, group B, scheduled euthanasia at 2 dpi, respiratory mucosa. The respiratory epithelial cells which can be identified based on their ciliated apical border (arrow) and the submucosal nerve fascicles (arrowhead) display no influenza A virus-matrixprotein immunoreactivity. **(C)** Chicken, P17-887, group B, scheduled euthanasia at 2 dpi, caecum. The caecal crypts extend deeply into the lymphoreticular tissue of the caecal tonsil and are lined by a columnar epithelium with typical brush border (arrow). **(D)** Chicken, P17-887, group B, scheduled euthanasia at 2 dpi, caecum. The lymphoreticular tissue of the caecal tonsils as well as the mucosa of the caecal crypts display no influenza A virus-matrixprotein immunoreactivity. **(E)** Chicken, P17-885,

group B, scheduled euthanasia at 2 dpi, brain. Neurons and glia within the brain show no obvious pathological alterations. **(F)** Chicken, P17-885, group B, scheduled euthanasia at 2 dpi, brain. There is no influenza A virus-matrixprotein immunoreactivity within the brain. **(G)** Chicken. P17-887. group B scheduled euthanasia at 2 dpi, spleen. A small artery (arrowhead) is surrounded by small differentiated lymphocytes with round,, heterochromatic nuclei (arrows) forming the periarteriolar lymphoid sheath. **(H)** Chicken, P17-887, group B scheduled euthanasia at 2 dpi, spleen. There are no influenza A virus-matrixprotein immunoreactivity cells within the lymphoreticular tissue of the spleen. A. C. E. G: Hematoxylin eosin. B. D. F. H: Influenza A virus-matrixprotein IHC, avidin-biotin-peroxidase complex method, using as first antibody a murine monoclonal antibody directed against the matrixprotein of anti-influenza A virus (strain PR8 (A/PR/8/34. H1 N1); clone M2-1C6-4R3 (ATCC® HB-64™). American Type Culture Collection. Manassas. USA), 3-amino-9-ethyl-carbazol as chromogen and hematoxylin counterstain. A-F: bar = 50 µm. G. H: bar = 20 µm.

Supplemental Figure 4a-f. Histopathological findings and virus tropism in chorioallantoic membrane and embryonal organs (supplement to Figure 4).

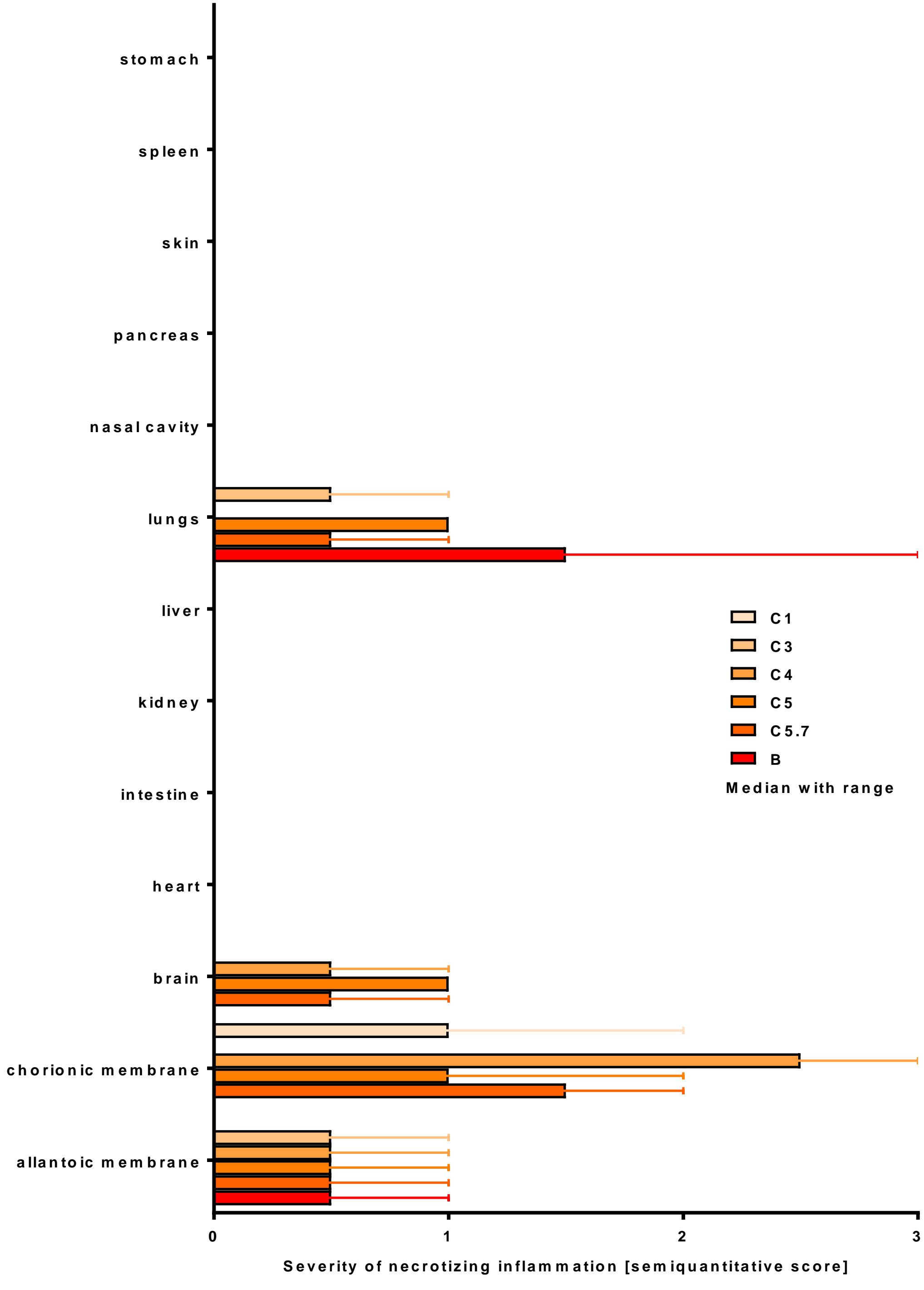
(a) 10-day old chicken embryos; Severity of necrotizing inflammation.

0 = negative; 1 = mild; 2 = moderate; 3 = severe.



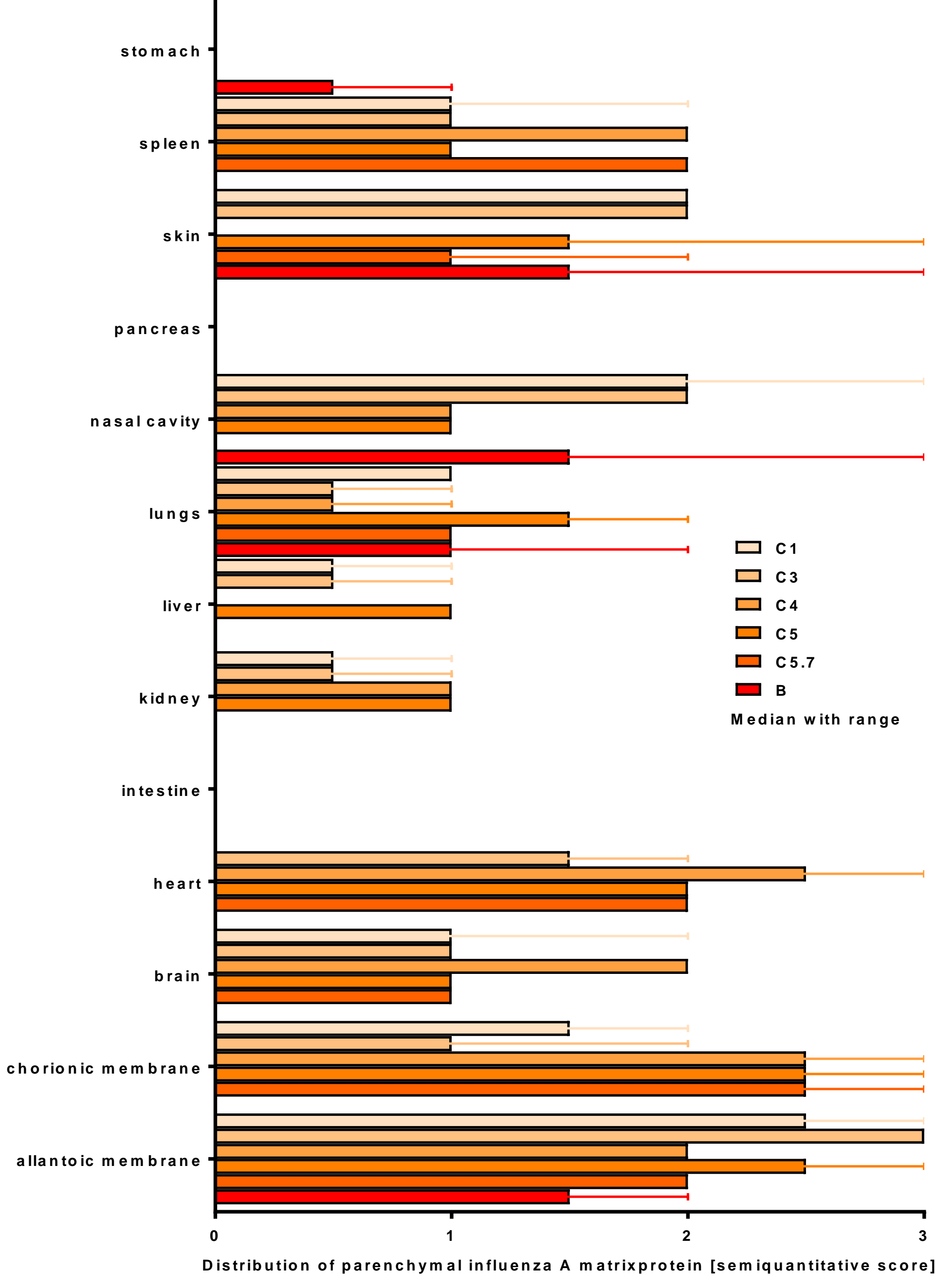
(b) 14-day old chicken embryos; Severity of necrotizing inflammation.

0 = negative; 1 = mild; 2 = moderate; 3 = severe.



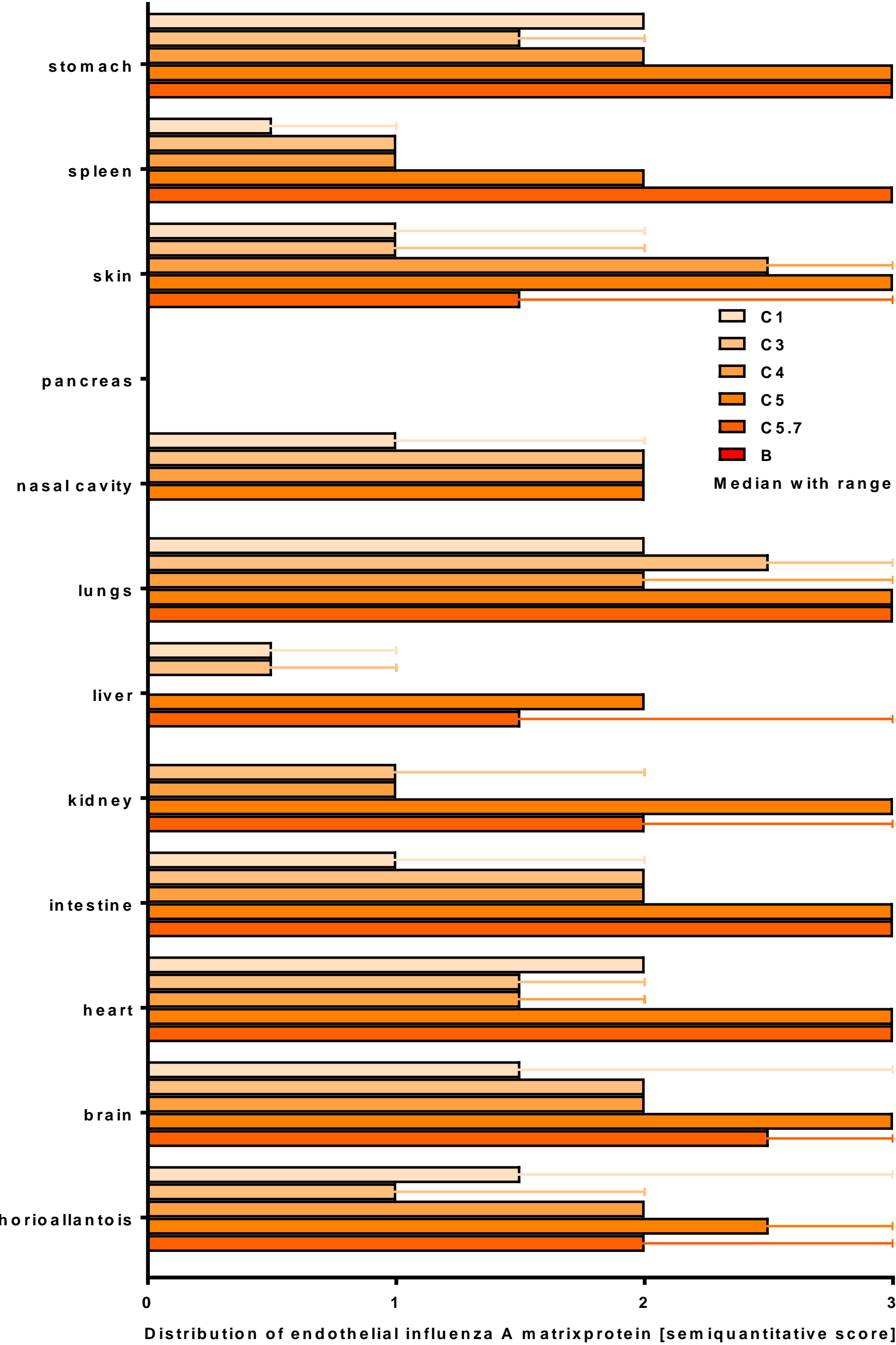
(c) 10-day old chicken embryos; Distribution of parenchymal and epithelial influenza A matrixprotein.

0 = negative; 1 = focal/oligofocal; 2 = multifocal; 3 = coalesing/diffuse.



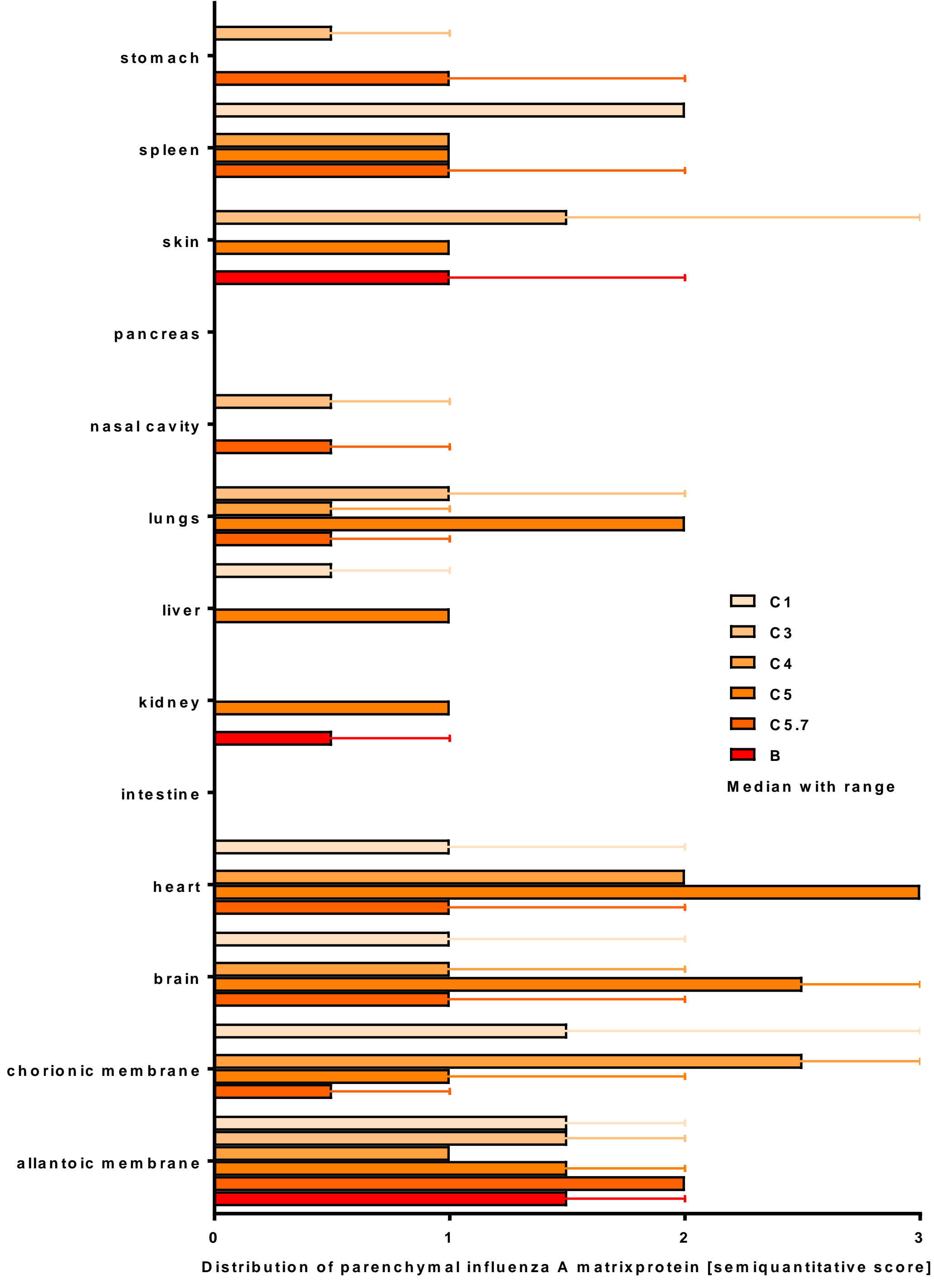
(d) 10-day old chicken embryos; Distribution of endothelial influenza A matrixprotein.

0 = negative; 1 = focal/oligofocal; 2 = multifocal; 3 = coalesing/diffuse.



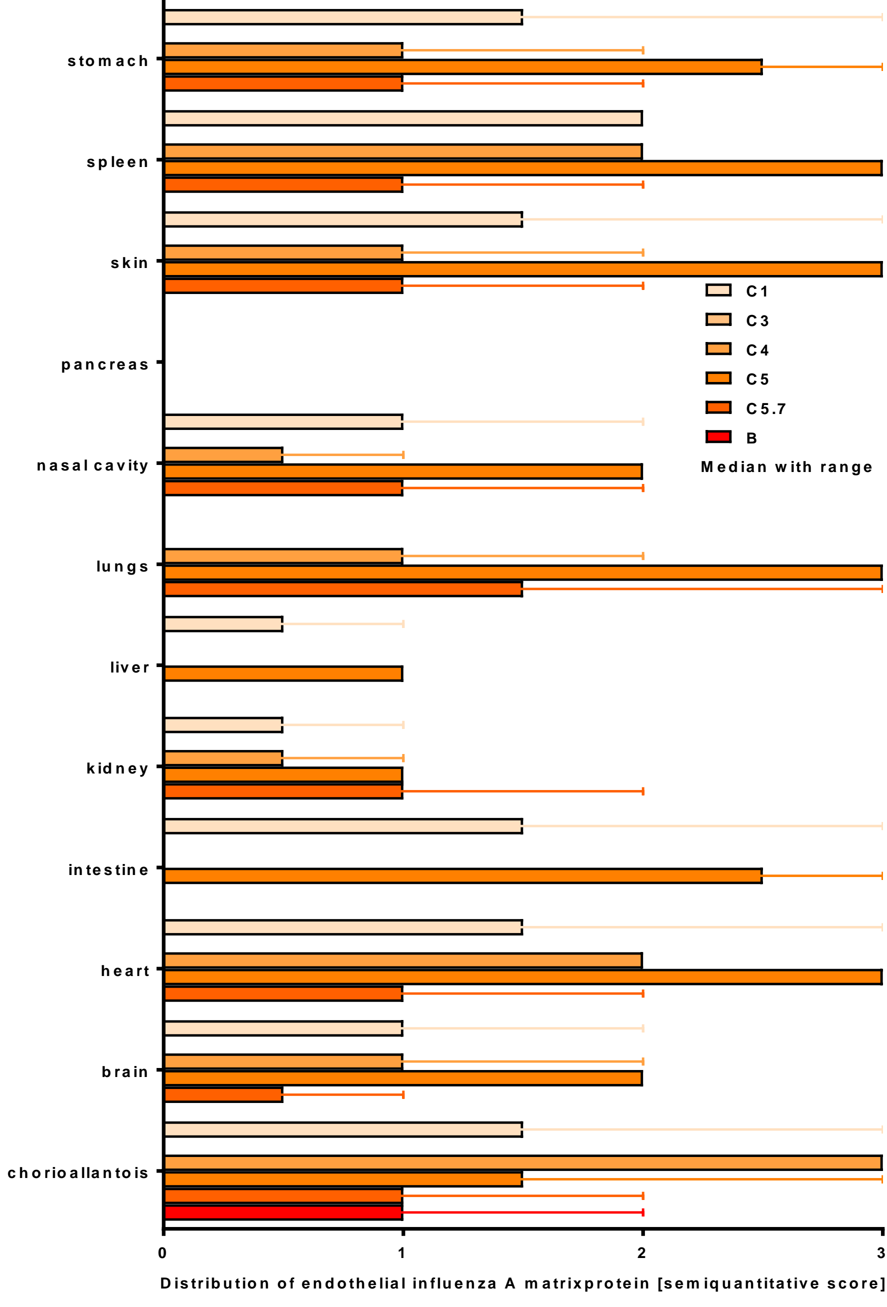
(e) 14-day old chicken embryos; Distribution of parenchymal and epithelial influenza A matrixprotein.

0 = negative; 1 = focal/oligofocal; 2 = multifocal; 3 = coalesing/diffuse.



(f) 14-day-old chicken embryos; Distribution of endothelial influenza A matrixprotein.

0 = negative; 1 = focal/oligofocal; 2 = multifocal; 3 = coalesing/diffuse.



Statistical analyses

The Mantel-Haenszel logrank test and the Mann Whitney test were used to compare survival rates and morbidity index as well as MDT values, respectively, applying the R software environment and the following packages: “stats”, “survival”, “survminer”, “gridExtra” and “ggplot2”. P values <0.05 were considered significant. For comparisons between the total amount of virus shedding of HPAIV in the mono- and co-infected groups, area-under-the-curve graphs were computed by using R software packages “stats”, “survival”, “survminer” and “ggplot2”. The mean average of Cq values of all animals sampled at the indicated dpi in a specific group were calculated and used to draw the curves. Animals negative in RT-qPCR at that date scored with a value of 40.

CHAPTER V: DISCUSSION

V. DISCUSSION

Over the past decades, both sporadic and enzootic outbreaks of notifiable LP as well as HP infections of AIV subtypes H5 and H7 continue worldwide causing devastating losses in poultry production. In some cases of zoonotic AIV, they also represent a significant public health problem by increasing the likelihood of sustained human-to-human transmission of avian-derived IAV which may spark a new human influenza pandemic (FAO, 2018; Kim et al., 2016; Peiris et al., 2007; Zanin et al., 2017). The most compelling approach to curtail such pandemic threats is to effectively control AIV in poultry (Peiris et al., 2016). In this line, the present study was designed to improve crucial steps in the diagnostic algorithm for subtypes H5 and H7 and to achieve a better understanding of the processes involved in the emergence of HPAIV from LPAIV precursors of these subtypes.

5.1 Diagnostic challenges – Developing improved tools for more effective surveillance (Study objective 1; chapters 4.1, 4.2, pp. 39-52, 53-66)

Apart from preventive measures, including in particular heightened biosecurity for poultry holdings, thorough surveillance and early detection of virus incursions are the main prerequisites for timely control. Swift diagnosis with rapid pathotype classification is of utmost importance to arrange for pondered, appropriate notification and control measures. Along with intravenous pathogenicity testing (IVPI) by an animal experiment, pathotyping of AIV is classically based on the nucleotide sequence analysis of the hemagglutinin cleavage site (HACS) encoding either single- (LP) or multibasic (HP) amino acid (aa) sequences using time consuming Sanger sequencing.

Here, rapid sequencing-independent assays targeting the HACS were developed to distinguish LP and HP variants of subtypes H7 and H5, respectively, by RT-qPCRs (chapters 4.1 and 4.2). The performance characteristics of these RT-qPCRs were determined to be closely similar to the generic M-specific PCR (Hoffmann et al., 2010), and PCR pathotyping of field samples led to the same results obtained with nucleotide sequencing. Nonetheless, the introduced pathotyping PCRs cannot be used generically but, owing to the sequence variability at the HACS especially of HP H7 viruses (see list of HACS motifs obtained from

outbreaks around the world published by the "OIE FAO network of expertise on animal influenza" (OFFLU et al., 2018)), require tailoring especially of the probe sequence to the respective strains in circulation. In cases when probe mismatches interfere with real time detection, nucleotide sequencing is still possible to confirm the pathotype by using the amplificates generated with the broadly reacting primers of these RT-qPCRs (chapter 4.1). A similar method and conclusions for RT-qPCR-directed pathotyping for gs/GD H5 viruses of clade 2.2 had been published earlier (Gall et al., 2009). Based on the pathotyping RT-qPCRs, we here propose an updated workflow for the molecular diagnosis of HPAIV H5 or H7 circulating in Europe: Firstly, the generic M-gene-specific RT-qPCR by Hoffmann et al. (2010) confirms the presence of influenza A viral RNA; several additional IAV generic RT-qPCRs can be used alternatively or in addition (M - Nagy et al. (2010), NP - Fereidouni et al. (2012), PB1 –Grund et al. (2018)). Secondly, subtype identification (for H5 and H7) using specific subtyping RT-qPCRs which are also embedded in the so called "RITA" (Riems influenza A typing array) is conducted (Hoffmann et al., 2016). If positive for H5 or H7, pathotyping is carried out by use of the newly developed pathotype-specific RT-qPCRs (chapter 4.1 and 4.2).

5.2 How HPAIV emerge – Investigating the relationship of LPAIV precursor and HPAIV effector viruses using newly developed diagnostic tools (Study objective 2; chapter 4.3, pp. 67-91)

There is convincing evidence from outbreaks and *in vitro* experiments that suggest HPAIV evolves from subtype H5 and H7 LP precursor viruses once introduced from wild birds into poultry (Alexander, 2007; Banks et al., 2001; Bean et al., 1985; Berhane et al., 2009; Capua et al., 2000a; Garcia et al., 1996; Perdue et al., 2003; Rohm et al., 1995; Rojas et al., 2002). Wild birds, especially aquatic birds act as reservoirs for AIV (Webster et al., 1992a). AIV mutation rates in chickens seem to be higher than in wild birds but it is unknown whether this and/or other factors also increase the likelihood of LP-to-HP mutations in this species (Fourment et al., 2015). For these reasons, all LPAIV H5 and H7 infections in poultry are notifiable and subject to statutory control as if HPAIV had been detected already. On-the-spot-evidence of spontaneous LP-to-HP mutations in the field is rare (Iglesias et al., 2010; Killian et al., 2016;

Monne et al., 2014; Seekings et al., 2018) or failed (de Wit et al., 2010a), and in most cases the precise source of LP introduction had been difficult to identify.

Here, a matching LP/HPAI virus pair was identified in specimens obtained from an H7N7 outbreak in two neighboring layer chicken farms in Germany in 2015 (chapter 4.3). The newly developed pathotyping RT-qPCRs in concert with next-generation sequencing (NGS) aided in confirming the emergence of the HP variant in a single epidemiological unit of the second farm following transmission of the LP variant from the first holding (chapter 4.3).

Mutation from LP to HPAIV appears to be governed by both viral and host-specific factors including host species, age, the immunological status of the host, genetic and environmental pressures etc. (Capua et al., 2013a; Vandegrift et al., 2010). The presence of an MBCS in the HA protein remains the main genetic determinant of HPAIV (Horimoto et al., 1994a; Klenk et al., 1975; Soda et al., 2011a). In the case of the German LP/HP H7N7 matching virus pair, the insertion of six nucleotides at the HACS as well as a single transitional nucleotide substitution were sufficient to convert the HACS from a single- to a multibasic pattern (chapter 4.3). Most probably, a stuttering mechanism of the viral replication complex when copying the sequence encoding the SBCS during antigenome and/or genome replication is at the basis of the insertional mutations of untemplated A and G residues, which finally translate into an MBCS (Pasick et al., 2005).

Experimental *in vitro, in vivo* and *in ovo* induction of LP-to-HP mutations by serial passaging of LPAIV or genetically engineering of an MBCS into the HA of LPAIV have been conducted and were successful in converting LPAIV to HPAIV in several but not all attempts (Abdelwhab et al., 2013; Abdelwhab et al., 2016a; Abdelwhab et al., 2016b; Abolnik et al., 2009; Banks et al., 2001; Bottcher-Friebertshauser et al., 2014; Brugh, 1988; Gohrbandt et al., 2011; Horimoto et al., 1994a, 1995a; Howard et al., 2007; Ito et al., 2001; Khatchikian et al., 1989; Li et al., 1990; Munster et al., 2010; Orlich et al., 1994; Orlich et al., 1990; Schrauwen et al., 2011; Soda et al., 2011b; Stech et al., 2009; Veits et al., 2012). However, presence of a MBCS was not always associated with a HP phenotype indicating that other viral factors within the HA gene segment or other gene segments have an influence on the HP phenotype expression (Abolnik et al., 2009; Londt et al., 2007).

NGS technology enables a sensitive detection of variants in quasispecies genomes of

influenza A viruses (Monne et al., 2014; Van den Hoecke et al., 2015), and thus depicts diversity in viral populations more accurately compared to Sanger sequencing methods. In our study (chapter 4.3, page 69), presence of mutations distinguishing LP from HP H7N7 outside the HACS were found by NGS as minor variants already in the LP progenitor quasispecies. We suggested that the emergence of the HP phenotype requires both, a selection of variants of the inner genome segments already present in the LP quasispecies population and a subsequent mutational shift in the HACS triggered by a stuttering RdRp. A similar mechanism has been furnished for the emergence of HPAIV H7N1 from LP precursor viruses during the Italian epizootic in 1999-2001 (Monne et al., 2014).

It has been proposed that the RNA secondary structure immediately distal of the HACS may be involved in fostering RdRp stuttering (Nao et al., 2017), and a similar stem loop structure was also identified in the LP precursor virus in our study (chapter 4.3, page 70). However, in our analyses, no differences in this part of the genome were seen between LP viruses that eventually yielded a HP mutant or did not give rise to such phenotype (chapter 4.3). Moreover, limited published data is available on the characterization of viruses possessing a di-basic CS (DBCS or also referred to as “mid-length” or intermediate HACS) (Luczo et al., 2018; Seekings, 2017). These studies speculated whether such DBCS is a requirement for further stepwise mutations towards a full MBCS since a DBCS has been shown to contribute to enhanced cleavage of HA, resulting in increased pathogenicity for poultry (Bashiruddin et al., 1992; Metreveli et al., 2010). No DBCS, however, has been identified in the quasispecies of our LP and HPAIV H7N7 viruses.

In addition to the HACS, other sides within the HA protein but outside the HACS were shown to enhance pathogenicity in poultry (Abdelwhab et al., 2013; Bogs et al., 2010; Diederich et al., 2015; Long et al., 2013; Post et al., 2013; Stech et al., 2009; Veits et al., 2012; Wang et al., 2010). For instance, the HP phenotype may be possibly modulated by an interrelationship between the number of N-linked glycosylation sites on the HA1 and NA stalk length (Matrosovich et al., 1999). Both mechanisms are believed to facilitate adaptation to gallinaceous poultry (Banks et al., 2001; Byrd-Leotis et al., 2017; de Wit et al., 2010b), but ultimate proof is lacking whether such factors are directly related to an increase in pathogenicity (Banks et al., 2003; Banks et al., 2001; Fouchier et al., 2004; Jonges et al., 2011).

Thus, no predictive markers are currently available nor could they be deduced from our study that would aid in assessing the risk of a certain LP H5/H7 virus to mutate to the HP phenotype. Deeper insights into the mutation processes and pathomechanisms governing the emergence of HPAIV would help to develop improved risk-based intervention strategies for notifiable LPAIV infections. So far, restriction measures must be taken for all LP H5 and H7 infected poultry (e.g. slaughter or culling of birds). Availability of quantifiable prognostic markers rendering a given LPAIV unlikely to progress to the HP phenotype would prevent culling of the affected flock. Although our pathotyping RT-qPCRs are a significant step forward in detecting LP/HPAIV variants, no such predictive markers emerged from our study.

5.3 “The winner takes it all” - Understanding the early processes after *de novo* emergence of HPAIV from LP precursor viruses (Study objective 3, chapter 4.4, pp. 93-138)

Understanding the patterns and dynamics of HPAIV emergence from a poultry population in which LPAIV is circulating, its transmission to other birds and further spread within flocks is important to select appropriate counteracting measures. In order to identify conditions that favor the spread and transmission of HPAIV over LPAIV and to understand how a minority of HP virions after *de novo* generation in a single host might gain primacy over LPAIV, co-infection studies in chapter 4.4 were initiated employing again the pathotyping tools of chapter 4.1 and the natural LP/HP matching virus pair of chapter 4.3. We attempted to mimic the initial moments of HPAIV emergence after *de novo* generation within an individual bird and in poultry population, respectively, that experiences concurrent infection by an antigenically identical LP precursor virus, as was the case in the layer hen flock from which our LP and HP H7 variants originated (chapter 4.3).

In the individual bird or ECE, direct and immediate interference between LP and HPAI H7N7 viruses was evident. HPAIV replication and, hence, its spread in the infected individual bird and excretion via the oropharynx and cloaca was severely hampered in the presence of an at least 100fold excess of antigenically identical LPAIV (chapter 4.4, page 91). This was confirmed both in co-infected chickens and ECEs. The factors responsible for the interference have not been studied here, but it seems likely that innate immune mechanisms such as interferon activity play a role (Tanikawa et al., 2017). In order to “avoid”

such LPAIV excess and to allow the HP phenotype to gain primacy, we assumed that LP–to-HP mutations should occur very early after LPAIV infection. Transmission from the index bird in which the HP phenotype emerged is not limited to oropharyngeal and cloacal secretions: due to the systemic course of HPAIV infection, infectious virus has been shown to be present in a multitude of tissues including muscle, feather cones, and eggs. Therefore, ingestion after picking on such tissues or mechanical transmission by haematophagous ectoparasites may be sufficient (Bertran et al., 2011; Sommer et al., 2016; Uchida et al., 2016; van den Brand et al., 2015; Yamamoto et al., 2017).

Since the LPAIV precursor-specific immunity just like immunization with a perfectly matching modified live virus vaccine effectively reduces susceptibility to homologous HPAIV infection and decreases HPAIV transmission efficacy (Nickbakhsh et al., 2016; Seo et al., 2001a; Van der Goot et al., 2003; van der Goot et al., 2005), spread in flocks of HP mutants after escape from an index bird depends on the presence of hosts that remain susceptible, i.e. non-exposed to the LP variant.

Therefore, at poultry flock level, the emerging HP phenotype would profit from a low prevalence of LP-specific adaptive immunity to avoid specific interference through adaptive immunity, and a low incidence of active LP precursor virus infection to prevent direct competition. Populations showing high seroconversion rates pose a low risk from an animal-disease-control perspective.

Our *in ovo* studies mirrored similar hampering effects of LPAIV infection on the replication kinetics of HPAIV co-infected embryos. In addition to this, the *in ovo* model further verified pathotype definitions such as endotheliotropism, a characteristic of HPAIV (Feldmann et al., 2000; Horimoto et al., 1998; Rott et al., 1980).

Considering all the obstacles that impede HP variants to gain primacy over its LP precursor, the *de novo* emergence of HP viruses at flock level is likely a very rare event. Nevertheless, several such HPAIV outbreaks have been described during the last two decades. It is tempting to speculate that conversely, many further LP-to-HP mutations may have remained undetected as the HP variants remained captured in the index birds or failed to spread in the population. Hence, it would be interesting to screen more LPAIV-positive field samples from regions where LPAIV has been circulating using our newly developed tools (chapter 4.1, 4.2)

and NGS technology to see whether HACS conversion events have occurred silently. In this line, the ECE infection model may be useful to study the LP-to-HP mutation frequency of different LPAIV *in ovo*.

5.4 Outlook on improved control of AIV in Europe and worldwide

Early-warning systems essentially require swift diagnosis facilitated by our newly developed diagnostic tools. These help to speed up measures aimed at repressing the spread of (potentially zoonotic) notifiable LPAIV and HPAIV. The relevance and versatility of these RT-qPCRs has been proven during HPAIV H7 emergence (chapter 4.1), continuing HPAIV H5 outbreaks (chapter 4.2) in the field as well as in pathogenesis research (chapters 4.3 and 4.4). As risks of new incursions into poultry are perpetuating due to the annual presence of LPAIV subtypes in wild bird populations, a long-term strategy with clearly defined goals is required. Essential steps consist of

1) intensification of syndrome surveillance to provide targeted, appropriate and timely intervention in LPAI infected poultry. Thus, the spread of an emerging virus with altered clinical and pathological as well as zoonotic potential must be prevented.
2) continuing adaptations and further developments of diagnostic tools.
3) depopulation of infected premises of all birds, once AI infection with notifiable subtypes H5 and H7 is confirmed. Financial compensation offered to poultry farmers is essential to build trust between the authorities and the poultry keepers.
4) However, due e.g. to limited financial resources, a stamping out policy alone cannot be used in endemically infected countries.
5) a "one health" approach including unified efforts from both veterinary and public health authorities for surveillance at the animal-human interface. Close contacts at the human-animal interface should be mitigated to decrease potential human exposure as well as possible subsequent human-to-human transmission. Public awareness concerning biosafety measures is mandatory.
6) reduction of transport of live poultry. This bears enhanced risks of virus spread. Biosecurity measures and poultry farming procedures must be tailored to the actual

epidemiologic situation. This includes wherever and whenever possible, housing of poultry indoor and situated away from open water sources to minimize possible interactions with wild birds.

7) the use of vaccination as a further tool depending on the epidemiological framework. It is important, however, that the pros and cons of a vaccination strategy are assessed individually beforehand. Vaccination alone is insufficient to eradicate AIV from an endemically infected population.

In general, these goals must all be flexibly selected and adapted according to epidemiology, poultry population structure and regional socio-cultural traditions.

CHAPTER VI: SUMMARY

VI. SUMMARY

Avian influenza viruses (AIV) are one of the most dominating and complex animal health threats in poultry production globally. Due to the zoonotic propensity of some AIV strain they may also have public health significance. According to their virulence in chickens, AIV can be distinguished into low pathogenic (LP) and highly pathogenic (HP) AIV. LPAIV of subtypes H5 and H7 have the ability to mutate into HP phenotypes through spontaneous insertional mutations in the hemagglutinin (HA) gene. The replication of LPAIV H5 or H7 in galliform poultry seems to be a prerequisite for the development of LP-to-HP mutations. There are continuing risks of new LPAIV incursions from the wild bird population as well as lateral spread of LP and HPAIV in domestic poultry and their re-transmission into wild bird populations. The continuing adaptation of appropriate diagnostic, control and prevention measures to meet viral evolution is of utmost importance. The work collected in this thesis presents the development of improved diagnostic methods for pathotyping. By using real-time quantitative polymerase chain reactions (RT-qPCRs) that characterize the HACS of AIV subtypes H5 and H7, we are capable to differentiate LP and HPAIV based on the HACS nucleotide sequences. They represent an alternative to animal experiments and sequencing-dependent pathotyping and thus aid in speeding up time-to-diagnosis and reduce reaction times of veterinary authorities in the context of notifiable outbreak infection events. In addition to this, the suitability of the newly developed diagnostic tools as a routine method in the field at the population level was applied in molecular-epidemiological follow-up studies of a combined LP/HPAIV H7N7 outbreak in two laying hen holdings in Germany in 2015. Using the H7 pathotyping RT-qPCRs, detection of LP/HP co-infections in swab specimens of the HPAI outbreak farm was achieved and, along with full-genome sequencing and H7-specific seroconversion of parts of the chicken population, a LP progenitor virus and its *de novo* mutant HP successor were demonstrated in the field. Finally, the new pathotyping diagnostic tools also proved advantageous in pathogenesis research on the spread of LP and HPAI viruses in tissues of experimentally co-infected chickens and embryonated chicken eggs (ECE). For this purpose, the recently detected natural LP/HP H7N7 pair of viruses was used to mimic the development and amplification of HPAIV mutants in LPAIV-infected animals. Chickens or ECE were co-infected with a constant

amount of LPAIV and increasing HPAIV titres. Pathotype-specific RT-qPCRs were used to demonstrate that LP co-infection had a significant inhibitory effect on HP H7 replication, viral excretion kinetics, and viral transmission to non-infected contact animals. Clinical, immunohistological and serological data confirmed these observations and revealed that HP variants arising *de novo* in an animal already infected with the antigenically identical LP precursor have to overcome obstacles related to direct viral interference, innate and adaptive immunity in order to be spread to other chickens. The methods developed here add an important optimization to the diagnosis of AI infections in birds. In addition, they contribute as tools in research to a deeper understanding of basic processes in the *de novo* generation and spread of HPAIV from LPAIV precursors. Nevertheless, the described diagnostic tests have limitations and are intended to be used mainly in screening programs, especially in regions with longer-term AI infection waves. When it comes to the initial recognition and characterization of new AIV outbreaks, the new pathotyping RT-qPCRs may need to be adapted based on the specific CS sequence of the actual virus strain in circulation.

CHAPTER VII: ZUSAMMENFASSUNG

VII. ZUSAMMENFASSUNG

Aviäre Influenzaviren (AIV) stellen eine der dominantesten und komplexesten Tiergesundheitsgefahren in der weltweiten Geflügelproduktion dar und können aufgrund ihres teilweise zoonotischen Charakters auch Bedeutung für die öffentliche Gesundheit erlangen. Nach ihrer Virulenz in Hühnern werden niedrig- (LP) und hoch pathogene (HP) AIV unterschieden. LPAIV der Subtypen H5 und H7 können durch spontane insertionelle Mutationen im HA Gen zu HP Phänotypen mutieren. Die Replikation von LPAIV H5 bzw. H7 in galliformen Hausgeflügel scheint eine Voraussetzung für das Entstehen der LP-zu-HP Mutationen darzustellen. Es bestehen kontinuierliche Risiken neuer LPAIV Einträge aus der Wildvogelpopulation sowie der lateralen Verbreitung einmal entstandener HPAIV in Hausgeflügel und deren Rückübertragung in Wildvogelpopulationen. Die stete Anpassung geeigneter Diagnose-, Kontroll- und Präventionsmaßnahmen an die virale Evolution ist von äußerster Wichtigkeit. Die in dieser Dissertation zusammengefassten Arbeiten betreffen die Entwicklung verbesserter diagnostischer Verfahren zur Pathotypisierung. Mithilfe von quantitativen Echtzeit-Polymerase-Kettenreaktionen (RT-qPCRs), welche die Hämagglutinin Spaltstelle von AIV der Subtypen H5 und H7 charakterisieren, ist es möglich, die LP- und HP-typische Nukleotidsequenzen in diesem Bereich zu differenzieren. Sie stellen eine Alternative zur tierversuchs- und sequenzierungs-abhängigen Pathotypisierung dar und beschleunigen somit die Diagnose und reduzieren die Reaktionszeiten im Rahmen anzeigepflichtiger Infektionsgeschehen. Außerdem konnte die Eignung der entwickelten diagnostischen Tests als Routinemethode im Feld auf Populationsebene durch Anwendung in der molekular-epidemiologischen Aufarbeitung eines LP/HPAIV H7N7 Ausbruchsgeschehens in zwei Legehennenbeständen in Deutschland im Jahr 2015 belegt werden. So gelang der Nachweis eines LP-Vorläufervirus und dessen mutierter HP-Version in Tupferproben der HPAI Ausbruchsfarm mittels der pathotypspezifischen RT-qPCR, Vollgenomsequenzen und H7-spezifischen serologischen Tests. Ein weiterer Einsatz der neuen diagnostischen Verfahren ergab sich in der Pathogeneseforschung zur Ausbreitung von LPAIV und HPAIV in Geweben experimentell infizierter Hühner und embryonierten Hühnereiern. Dazu wurden Ko-Infektionen unter Verwendung des beschriebenen natürlichen LP/HP H7N7 Viruspaares durchgeführt. Hierdurch sollte die Entstehung von HP

Mutanten in mit LP Virus infizierten Tieren simuliert werden. Hühner bzw. embryonierte Hühnereier wurden mit einer konstanten LPAIV-Inokulumdosis und steigenden HPAIV Titern ko-infiziert. U.a. mittels pathotyp-spezifischer RT-qPCRs konnte gezeigt werden, dass die LP-Ko-Infektion einen signifikant hemmenden Einfluss auf die HP H7-Replikation, die virale Ausscheidungskinetik sowie die Virusübertragung auf nicht infizierte Kontakttiere zur Folge hatte. Klinische, immunhistologische und serologische Daten bestätigten diese Beobachtungen. Es konnte gezeigt werden, dass HP-Varianten, die *de novo* in einem bereits mit dem antigenetisch identischen LP-Vorläufer infizierten Tier entstehen, Hindernisse in Bezug auf direkt virale Interferenz, angeborene und adaptive Immunität überwinden müssen, um auf andere Hühner überzutragen zu werden.

Die hier erarbeiteten Methoden stellen eine wichtige Optimierung der Diagnostik von AI Infektionen dar. Darüber hinaus konnten die neuen Techniken als Forschungs-Werkzeuge hin zu einem tieferen Verständnis grundlegender Prozesse in der *de novo* Entstehung von HPAIV aus LPAIV Vorläufern beitragen. Die beschriebenen diagnostischen Tests weisen dennoch Limitationen auf und sind hauptsächlich für den Einsatz in Screening-Programmen, insbesondere in Regionen mit längerfristig ablaufenden AI-Infektionswellen, vorgesehen. Wenn es um die Ersterkennung und -charakterisierung neuer AIV-Ausbrüche geht, müssen die neu entwickelten Tests ggf. anhand der spezifischen Spaltstellensequenz adaptiert werden.

CHAPTER VIII: REFERENCES

VIII. REFERENCES

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CHAPTER IX: SUPPLEMENT

IX. SUPPLEMENT

1. LIST OF ABBREVIATIONS

Aa	Amino acid
AI	Avian influenza
AIV	Avian influenza virus
BLAST	Basic local alignment search tool
cRNA	complementary ribonucleic acid
CPSF30	Cleavage and polyadenylation specificity factor 30
CS	Cleavage site
Ct	Cycle threshold
Cq	Cyle of quantification
ds	double-stranded
Dpi	Days post infection
DNA	Deoxyribonucleic acid
ECE	Embryonated chicken egg
EFE	Embryonated fowl egg
EID50	50% egg infectious dose
FAO	Food and Agriculture Organization of the United Nations
FLI	Friedrich-Loeffler-Institute
GISAID	Global initiative on sharing all influenza data
Gs/GD	Goose/Guangdong
HA	Hemagglutinin
HACS	Hemagglutinin cleavage site
HAU	Hemagglutinating units
HI	Hemagglutinin inhibition assay
HP	Highly pathogenic
HPAI	High pathogenic avian influenza
HPAIV	High pathogenic avian influenza virus
Hpi	Hours post infection
IAV	Influenza A virus

IHC	Immunohistochemistry
IVPI	Intravenous pathogenicity index
K	Lysine
Kbp	Kilo base pair
LBM	Live bird market
LP	Low pathogenic
LPAI	Low pathogenic avian influenza
LPAIV	Low pathogenic avian influenza virus
M	Matrix gene
MB	Multi-basic
MBCS	Multi-basic cleavage site
MDT	Mean death time
mRNA	messenger RNA
NA	Neuraminidase
NCBI	National center for biotechnology
NEP	Nuclear export protein
NGS	Next generation sequencing
NP	Nucleoprotein
NS	Non-structural protein
Nt	Nucleotide
2´-5´OAS	2´-5´ oligoadenylate synthetase
OFFLU	OIE/FAO Influenza Network
OIE	World Organization for Animal Health
PA	Polymerase acidic protein
PABPII	polyadenine binding protein II
PB1	Polymerase basic-1 protein
PB2	Polymerase basic-2 protein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
R	Arginine
RIG-I	Retinoic acid-inducible gene I

RBD	Receptor binding domain
RBS	Receptor binding site
RdRp	RNA-dependent RNA-polymerase
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RNA	Ribonucleic acid
RT-qPCR	Quantitative real time RT-PCR
SA	Sialic acid
SBCS	Single basic cleavage site
SPF	Specific pathogen free
ss	single-stranded
TCID	Tissue culture infectious dose
vRNA	viral ribonucleic acid
vRNP	viral ribonucleoprotein
WHO	World Health Organization

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CHAPTER X: ACKNOWLEDGMENT

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