Genetic changes that accompanied shifts of low pathogenic avian influenza viruses toward higher pathogenicity in poultry

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Abbreviations: AIV, avian influenza viruses; BC, British Columbia; HA, hemagglutinin; HP, highly pathogenic; IVPI, intravenous pathogenicity index; LBM, live bird markets; LP, low pathogenic; M, matrix; NA, neuraminidase; NEP, nuclear export protein; NP, nucleoprotein; nt, nucleotide(s); NS, non-structural; PA, acidic polymerase; PB, basic polymerase; PCS, proteolytic cleavage site; RBD, receptor binding domain; SA, sialic acid; SK, Saskatchewan

Avian influenza viruses (AIV) of H5 and H7 subtypes exhibit two different pathotypes in poultry: infection with low pathogenic (LP) strains results in minimal, if any, health disturbances, whereas highly pathogenic (HP) strains cause severe morbidity and mortality. LPAIV of H5 and H7 subtypes can spontaneously mutate into HPAIV. Ten outbreaks caused by HPAIV are known to have been preceded by circulation of a predecessor LPAIV in poultry. Three of them were caused by H5N2 subtype and seven involved H7 subtype in combination with N1, N3, or N7. Here, we review those outbreaks and summarize the genetic changes which resulted in the transformation of LPAIV to HPAIV under natural conditions. Mutations that were found directly in those outbreaks are more likely to be linked to virulence, pathogenesis, and early adaptation of AIV.

Introduction

Avian influenza viruses (AIV) belong to the genus *Influenzavirus A* within the family *Orthomyxoviridae*, which contains five genera: *Influenzavirus A*, *B*, *C*, *Thogotovirus*, and *Isavirus*.[1] AIV, the only orthomyxovirus known to infect birds, are enveloped viruses which contain a negative-sense, single-stranded ribonucleic acid (RNA) multipartite genome of eight separate gene segments encoding at least 11 viral proteins.[2,3] The viral proteins have been allocated into three main categories: envelope proteins (hemagglutinin [HA], neuraminidase [NA], and matrix protein-2 [M2]), internal proteins (polymerase subunits [PB2, PB1, PA], nucleoprotein [NP], matrix protein 1 [M1], and nuclear export protein [NEP]) and non-structural proteins (NS1 and PB1-F2).[3-5] Of the two envelope glycoproteins HA and NA 16 HA (H1–H16) and 9 NA (N1–N9) subtypes have been distinguished

in AIV,[1,6] whereas recently a novel H17N10 virus has been identified in bats in Guatemala.[7] All AIV subtypes are present in wild aquatic birds, the natural reservoir, and transmit sporadically to poultry and/or mammals.[4] Host range of AIV is primarily determined by receptor binding preference. Avian viruses attach to α2-3 sialic acid linked galactose (SA) which is abundant in the alimentary tract of avian species, whereas mammalian including human influenza viruses prefer binding to α2-6 SA abundant in the human respiratory tract.[8,9] Three HA (H1, H2, and H3) and two NA (N1 and N2) subtypes resulted in pandemic infections in humans, but avian H5, H7, and H9 viruses also infected humans in several countries.[10]

Due to the viral error-prone RNA polymerase constant antigenic variation of AIV is an intriguing feature for continuous evolution of the virus in nature to evade the host's immune response and allow reinfection.[5] Gradual antigenic variation via incremental acquisition of point mutations is defined as "antigenic drift" which is commonly regarded as the driving mechanism for annual influenza virus epidemics. "Antigenic shift" of influenza virus occurs by exchange ("reassortment") of genes from different influenza subtypes leading to a complete change in the antigenic structure and emergence of new viruses[5,11,12] that may result in severe pandemics.[13,14] According to their pathogenicity in poultry, AIV is classified into two pathotypes. Low pathogenic (LP) strains result in mild or asymptomatic infections, whereas highly pathogenic (HP) strains cause up to 100% morbidity and mortality.[15] Any AIV that exhibits an intravenous pathogenicity index (IVPI) in 6-week-old chickens greater than 1.2 or kills at least 75% of 4- to 8-week-old chickens during a 10-day-observation period is defined as a highly pathogenic strain.[16-18] Moreover, all influenza A viruses are proteolytically cleaved within their HA protein at an amino acid sequence designated as the proteolytic cleavage site (PCS). This consists of an arginine (R) residue adjacent to a conserved glycine (G) which becomes the N-terminus of the HA_2 subunit.[19] There is a strong positive correlation between the number of basic amino acids in the PCS and an increase of virulence of AIV.[20-24] LPAIV usually
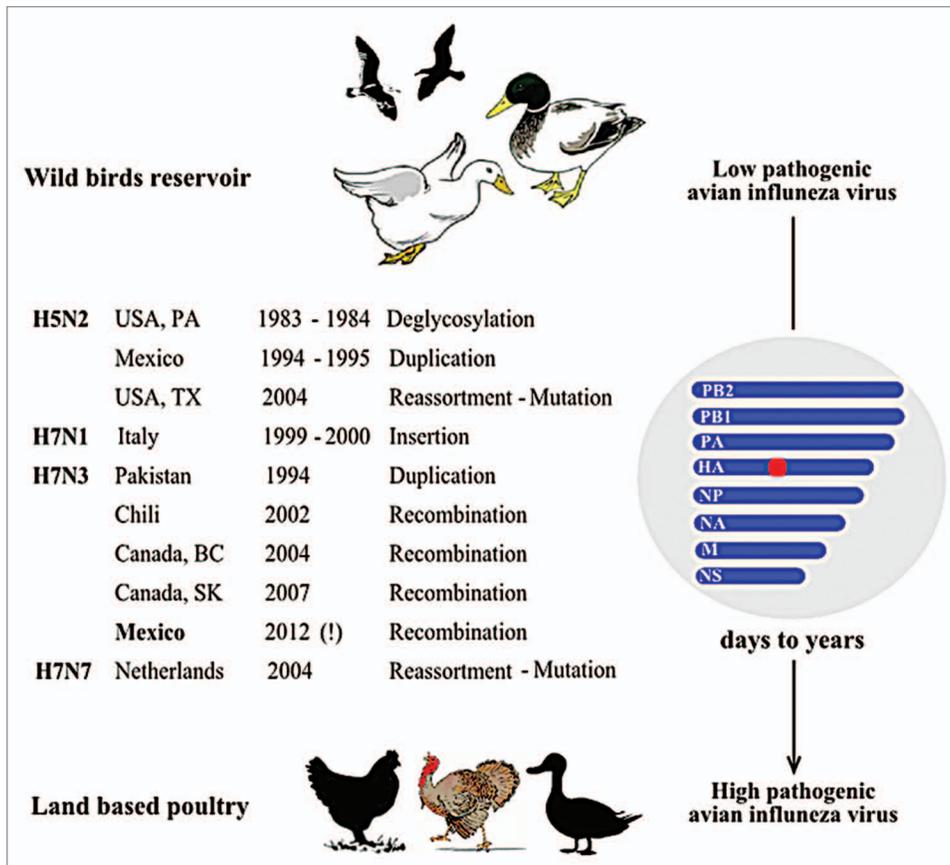
Figure 1. Major genetic changes found in HPAIV that have directly evolved from LPAIV. Shown are the genetic changes found in the hemagglutinin (particularly proteolytic cleavage site; denoted as a red square) that accompanied shifts of low pathogenic avian influenza viruses from wild birds reservoir toward higher pathogenicity in land based poultry. The transition periods ranged from few days to several years. All outbreaks were eradicated while the exclamation mark refers to the on-going H7N3 outbreak in domesticated poultry in Mexico since June 2012.

of AIV.[33,35-41] However, the steps by which LPAIV becomes HPAIV in nature remain poorly understood.

To date, ten outbreaks of HPAIV in poultry are documented in which the HPAIV isolated from birds in these outbreaks is thought to originate from an LPAIV previously circulating in the poultry population (**Fig. 1**). Three outbreaks were caused by H5N2 subtype and seven by H7 in combination with N1, N3, or N7 in 6 countries on 4 different continents. Genetic changes in HPAIV that occurred during acquisition of virulence under field conditions (**Table 1**) can give a molecular clue for better understanding the mechanism of AIV pathogenicity. Therefore, this review aims to provide an insight into the epidemiology and molecular aspects which accompanied the shift in virulence of AIV in natural outbreaks in poultry.

Epidemic of H5N2 in Pennsylvania, USA

LPAIV A/Chicken/Pennsylvania/1/83 (H5N2), which belongs to the North American lineage of wild bird AIV, was first reported in Pennsylvania in April 1983 at a chicken farm as the causative agent of an acute respiratory disease, caus-

exhibit a single basic amino acid, arginine (R) or lysine (K), at the cleavage site, while multiple basic amino acids with a minimal $^{-4}$R-X-K/R-R^{-1} motif are characteristic for HPAIV.[20,25] Two different classes of proteases are responsible for cleavage-activation of the hemagglutinin of influenza viruses and the distribution of these proteases in the host appears to be a prime determinant of AIV tissue tropism and pathogenicity. The trypsin-like proteases that cleave LPAIV are present only in a limited number of cells or tissues, so that these viruses normally cause localized infections in, for example, the respiratory tract of mammals or intestinal tract of birds. In contrast, furin and subtilisin-like proteases that activate HPAIV are ubiquitously expressed causing systemic spread of the virus.[11,26-29] To date, several strains of H5 or H7 subtypes fulfill the criteria of high pathogenicity. They emerge at irregular intervals and can devastate poultry flocks within a few days.[11,30-32] No avian or mammalian reservoirs for HPAIV have been identified yet but there are several well recorded examples of how they evolved from LPAIV.[16,33,34] Within the last three decades, the development of reverse genetic techniques helped researchers to correlate many genetic markers with virulence

ing 2.6% mortality and 31% drop in egg production.[42-44] During in vivo pathogenicity test, the virus did not produce clinical signs or mortality in experimentally infected chickens and replicated in vitro only in the presence of exogenous trypsin. Therefore, it was classified as LPAIV. Epidemiological investigations indicated that the virus was probably transmitted from wild waterfowl to the gallinaceous poultry.[42,45-47] In October 1983, an H5N2 virus was isolated from commercial chickens after complete cessation of egg production, exhibiting nervous signs and 50–89% mortality within few days. All experimentally infected white leghorn chickens died and the virus replicated well in the absence of trypsin, prompting its classification as HPAIV.[34,43,44] Thereafter, experimental in vitro, in ovo, and in vivo passages of the parental LPAIV H5N2 generated several different HPAIV variants.[23,31,48,49] The HPAIV H5N2 produced mild transient, if any, clinical signs after infection of pheasants, gulls, quails, and Pekin ducks.[45] The HPAIV H5N2 had also been isolated from pigs without further pig-to-pig transmission[34,43,44] and flies caught in the chicken houses.[34,43,44] It was also isolated from 2 out of 40 investigated workers involved in culling the infected

Table 1. Epidemiology and genetic changes of outbreaks caused by HPAIV that have directly evolved from LPAIV

Outbreak	History		Hosts		Amino acid substitutions												
	LPAIV	Time for transition (~month)	Birds	Mammals	PCS	PB2	PB1	PB1-F2	PA	HA	NP	NA	M1	M2	NS1	NEP	Total
Pennsylvania H5N2 (1983)	H5N2 04.1983	6	CK, TK, PH	2 workers & a pig	Lp: PQKKKR/GLF Hp: PQKKKR/GLF	V229I	E331K	0	D3N T151N	T13K E69D	0	0 Del. 53-82	I169T	L43F D44N	0	0	9
Mexico H5N2 (1994)	H5N2 05.1994	8	CK	NR	Lp: RETR/GLF KETR/GLF Hp: RKRKTR/GLF RKRKRKTR/GLF	I451T T471A V480I I539V A674T	V113I V149I S375N R486KI 535V	Q5L T36I R37L S83Y	I61K M86V G99R V118I A210T I308F A343T H346N D382E S402A I459L R508K A553S L683I	R40K L44I A86V S109N R234S D261E S287T D448N	M447I	V51I L235I Del. 53-82	L117F K230R	N13S F47L	R25K F138S/L	0	45
Texas H5N3 (2004)	H5N2 05.2002	24	CK	NR	Lp: PQREKR/GLF Hp: PQRKKR/GLF	UK	UK	UK	UK	D31N F70L G139S Q158R T231A N244T E404G	M136L A423T	Del. 51-63	R101 G110E	UK	P6I V65I A80S G211R V226A	0	16
Italy H7N1 (1999)	H7N1 03.1999	9	Many avian species*	Neither antibodies nor antigen	Lp: PKGR/GLF Hp: PKGSRVRR/GLF	T398I R508M	G154D S216G K586R K745E	0	I60T N115S E252K I459M	T112A A436T K536R	T349A S376N	R173K Del. 54-75	V166A	0	V136I D139N	0	19
Pakistan H7N3 (1995)	H7N3 12.1994	8	CK	NR	Lp: PKGR/R Hp: PKRKKR/GLF PKRKR/GLF PKRRNR/GLF	0	0	I18T	0	31 mutations	0	L84H	0	0	0	0	33
Chile H7N3 (2002)	H7N3 04.2002	1	CK, TK	NR	Lp: PKTR/GLF Hp: PKTCSPLSRCRETR/GLF PKTCSPLSRCRKTR/GLF	T16A Y111H R318G F330S V451A T482K N506D S653T R753G	T204I E387K G754R	K44R	I30T F95C L211M R378K G613E	A20T D123N T128A	M105I S132G	L392V	0	0	0	0	24
Canada (BC) H7N3 (2004)	H7N3 02.2004	11 days	CK	2 workers	Lp: PKTR/GLF Hp: PKQAYRKRMTR/GLF PKQAYQKRMTR/GLF PKQAYQKQMTR/GLF	0	0	0	0	0	0	0	0	0	0	0	0
Netherlands H7N7 (2003)	**H7**N3 (2003) H10**N7** (2000)	36	CK	86 workers & 3 family members	Lp: PKGR/GLF Hp: PKRRRR/GLF	UK	UK	UK	UK	(M-7V) E261G M490I	UK	S16A T42P E75G I126T G188A A267E K269R V390I S458P	UK	UK	UK	UK	12

*Turkeys (TK), chickens (CK), guinea-fowl, quail, pheasants (PH), ostriches, ducks, geese, sparrows, doves, and Saker-falcon. UK, unknown; NR, not reported. Numbering of the HA is conducted at the mature protein after removal of the signal peptide sequences, whereas numbering of other proteins begins at the start codon. Alignment of full genome segments and the accession numbers of LP and HP viruses are available up on request.

chickens when samples were collected immediately within 12 h after exposure to infected birds.[34,43,44]

Surprisingly, the PCS motifs of the low and highly pathogenic viruses were identical, specifying PQKKKR/GLF. Using reverse genetics, pathogenicity of the Pennsylvanian H5N2 viruses was linked to the HA gene, since a reassortant virus with the HA gene of the virulent strain and other gene segments from the avirulent strain killed all experimentally infected chickens.[50] It was subsequently detected that a mutation (T13K) in the HA of the virulent virus led to loss of an N-glycosylation consensus motif (asparagine) at position 11 which enhanced the cleavability of PCS by furin-proteases.[42,47,50,51] Interestingly, in vitro studies aimed to increase the number of basic amino acids in the PCS of the avirulent virus abolished the masking effect of glycosylation at position 11 and induced shift in the pathogenicity of the avirulent virus.[48] Furthermore, reassortant LPAI H5N1 and H5N9 viruses of shorebird origin containing the HA and M gene segments of the Pennsylvanian HPAIV exhibited increased virulence in chickens.[52] In addition, a 20 amino acid deletion in the NA stalk region (residues 63 to 82) was reported which is common for chicken-adapted AIV.[53]

Epidemic of H5N2 in Mexico

Early serologic evidence of circulation of an H5N2 influenza virus among Mexican chickens was reported in October 1993.[54] In May 1994, the LPAIV was isolated for the first time from several flocks nationwide suffering from mild respiratory disorders. Gradual increase in virulence of the H5N2 virus was observed in November 1994 in Puebla, Mexico. However, the virus still exhibited only mild virulence in chickens, since zero to 4 out of 8 intravenously infected chickens died.[54,55] However, by January 1995, the virus had become highly lethal for poultry and killed all experimentally infected chickens.[55] Based on the sequence and phylogenetic analysis of the HA it was stated that the Mexican virus belonged to the North American lineage introduced by

migrating waterfowl which evolved thereafter in the Mexican chicken population and that the HPAIV was derived directly from the LPAIV isolated approximately one month earlier.[54,56,57]

The PCS motif of the early LPAIV was PQR**E**TR/G (or more rarely PQ**K**ETR/G), a feature of avirulent viruses.[54,58] However, substitutions by and insertions of basic amino acids, PQR**KRK**TR/G (one isolate had PQR**KRKRK**TR/G[58]), due to strand slippage and a duplication in the PCS region were found in both mildly (isolated in November 1994) and highly pathogenic viruses (isolated in January 1995).[54,56,57] These changes enhanced HA cleavability. Existence of intermediate PCS (PQR**K**TR/G or PQR**KRE**TR/G) has been suggested.[54] Although the PCS was identical in mildly and highly pathogenic viruses, there was no remarkable difference in the cleavability of HA suggesting that virulence of these viruses was determined by factors beyond the PCS, which remain to be investigated.[54,55,57] Interestingly, after infection of chickens with mildly pathogenic viruses, lesions were restricted to few organs and birds probably died due to respiratory and/or renal failure. In contrast, HPAIV was pantropic in replication and death occurred due to failure of multiple vital organs particularly the heart and pancreas. This different tropism may be governed by other, yet undetermined genomic changes rather than the PCS.[34]

This HPAIV H5N2 epidemic in Mexico had been controlled by inactivated vaccines containing local seed virus and has not been reported since 1996, but LPAIV H5N2 remains endemic in Mexico and has spread to poultry in two other adjacent countries, Guatemala and El Salvador.[58]

Epidemic of H5N2 in Texas, USA

Presence of an LPAIV H5N3 in commercial chickens in Texas connected with live bird markets was first reported in May 2002.[59] It has been suggested that the virus was introduced by wild birds.[59-61] The Texas/H5N3 virus specified the PCS sequence PQR**E**KR/G,[59,62] but was classified as LPAIV based on the pathogenicity in chickens.[59] Experimental infections demonstrated that LPAIV Texas/H5N3 replicated efficiently in turkeys followed by chickens and ducks.[59,61,62] The virus induced signs of illness and spread to several organs in chickens beyond the trachea and intestine including the cerebrum.[59] It was assumed that this virus had been circulating in domestic chickens for some period of time before it was isolated from diseased commercial poultry. The Texas/H5N3 LPAIV had a 28 amino acid deletion in the NA stalk and all 6 potential glycosylation sites in the HA$_1$ protein but lacked the glycosylation at position 158, which is considered a genetic marker for adaptation to poultry.[9] Moreover, no HPAI evolved from this virus after six serial passages in experimentally infected chickens.[62]

On February 2004, an HPAIV H5N2 was confirmed in a broiler flock and 2 of 5 investigated live bird markets.[60,63,64] The affected birds showed only mild clinical signs resembling LPAIV infection. However, the deduced amino acid sequence in the PCS of Texas/H5N2 revealed that the virus had acquired one additional basic amino acid at the PCS (PQR**K**KR/GLF) complying with the definition of HPAIV. Nonetheless, the virus was not virulent for experimentally infected chickens (IVPI = 0.0).[63] It was found that Texas/H5N2 acquired HA, NP, M, and NS genes from Texas/H5N3, but the polymerase and NA genes were donated by another unknown virus.[63] Although investigation failed to elucidate why a virus with multibasic PCS remained mildly virulent for chickens, insertion of one or two additional basic amino acids at the PCS increased virulence both in vitro and in vivo.[63] It is worth pointing out that both Texas/H5N2 and its precursor had the same HA glycosylation pattern and conserved receptor binding sites. In contrast, Texas/H5N2 virus had only a 13 amino acid deletion (residues 51 to 63) at the NA stalk region.[63]

Epidemic of H7N1 in Italy

On 29 March 1999, LPAIV H7N1 was diagnosed and subsequently detected in 199 outbreaks in northern Italy to be marked as the largest epidemic of LPAIV reported in a Western country. Surveillance in wild ducks, gulls, and coot revealed absence of circulation of LPAIV H7N1 among tested wild birds prior to or immediately after emergence of the virus in domestic poultry.[65,66] However, other hosts might be implicated in its introduction[65] since the LPAIV clustered phylogenetically within a group of Eurasian and South African viruses of wild-bird origin.[67] After 9 mo, on December 13, an HPAIV of the same subtype emerged in multiple poultry species in a densely populated area[68,69] causing 413 reported outbreaks in turkeys, chickens, guinea-fowl, quail, pheasants, ostriches, ducks, geese, sparrows, doves, and Saker falcons[68-74] and, after experimental infection, killed a red-legged partridge.[75] The disease resulted in culling of more than 13 million birds, disrupted the poultry marketing system, and was eradicated within 5 mo.[32,68]

The LPAIV H7N1 had the monobasic PCS motif PKGR/GLF, while the subsequent HPAIV specified the multibasic PCS motif PKG**SRVR**R/GLF.[68,69] The progenitor LPAIV grew well in embryonated eggs obtained from mallard ducks, but not in chicken, turkey, and Muscovy duck embryos, whereas the HPAIV replicated well in all embryos.[71,76] All Italian AIV had a deletion of 22 amino acids (position 54 to 75) in the NA stalk region.[67] Recently, using reverse genetics, an Italian LPAI with a short-stalk-NA was generated and found to be more virulent for chicken than the long-stalk-NA mutant. In contrast, short-stalk-NA was found to be disadvantageous for a sustained virus replication and transmission in ducks.[77] The NS1 gene of the HPAIV showed a progressive C-terminal truncation resulting in a 6 amino acid deletion in the NS1 protein in addition to V136I and D139N substitutions. Truncation of the NS1 gene increased the lethality of the HPAIV for embryonated chicken eggs, whereas V136I and D139N substitutions increased the virulence of the LPAIV in mice.[78] Recently, LPAIV H7N1 with a six-amino-acid truncation within NS1 was created which did not influence virus replication in duck or chicken cells.[79] However, truncation of NS1 to 99 amino acids in length converted an Italian LPAIV into a strong interferon inducer in duck cells.[80] An Italian LPAIV H7N1 with a human C-terminal RSKV motif in NS1 increased viral replication in ducks and induced high levels

of the interferon-stimulated Mx gene, whereas the NS1 with the avian C-terminal ESEV motif increased virulence in mice causing severe pneumonia and a high level of type I interferon.[81]

Interestingly, infection was not recorded in any non-avian species including humans throughout the duration of the H7N1 outbreak in Italy.[82] Serological examination of 798 serum samples collected from persons with close contact with infected poultry yielded no evidence of infection, and viral RNA could not be detected in clinical samples tested by RT-PCR.[32,83] Yet, the Italian HPAIV H7N1 had the potential of interspecies transmission to mammals under experimental conditions. One ostrich-origin virus with 627K in the PB2 was lethal to experimentally infected mice and the virus has been recovered from the nervous and respiratory systems.[84,85] Moreover, chicken-origin virus which specified 627E in PB2 protein was able to mutate to 627K after only one passage in mice.[84,85] Different vaccines developed from Italian HPAIV H7N1 protected mice and ferrets against experimental infections.[86-88]

Epidemic of H7N3 in Pakistan

1994–1995 epidemic. First outbreaks of H7N3 were reported in broiler-breeder and broiler flocks in northern Pakistan from December 1994 to April 1995.[89] The virus was classified as LPAIV[89-91] and no vaccination had been implemented to control the disease at that time.[89] However, in 1995, after few months, the LPAIV mutated to HPAIV[92] which has been controlled within four months by enforcing biosecurity measures and vaccination with inactivated local-strain seeded vaccine.[89,90]

The precursor LPAIV H7N3 had the monobasic PCS motif PE**I**PKGR/R while multibasic PCS with PETPK**RKRK**R/GLF or PETPK**RRK**R/GLF (very rarely PETPK**RRN**R/GLF) was characteristic for the HPAIV.[91,93] These mutations occurred most likely by duplication in the PCS.[57] Interestingly, this LPAIV H7N3 had 89.5% nucleotides (nt) identity with the HA genes of the Pakistani HPAIV H7N3, whereas it showed 99.7% identity with the historic A/Parrot/North Ireland/YF73-67/1973 (H7N1) and up to 93% with recent viruses from poultry in China and Italy during 1997–2003 indicative of a probable common ancestor.[91] These extensive alterations, particularly in the HA$_1$ gene in Pakistani HPAIV H7N3 are surprising. A possible explanation for this anomaly could be a reassortment with another unidentified strain(s).[89] Moreover, NS1 and NS2 were truncated to respectively 216–217 and 120–121 amino acids, whereas the other genes of the Pakistani LPAIV and HPAIV H7N3 were identical.

2003–2004 epidemic. Isolation of LPAIV H7N3 from commercial layer flocks was reported from April to October 2003 in the coastal southern town of Karachi, where the majority of the Pakistani table-egg layer flocks were raised.[90] In November 2003 a devastating HPAIV H7N3 suddenly emerged and widely spread within few weeks to the broiler-breeder stocks in the Northern part of the country.[90] This outbreak lasted until June 2004 and was finally controlled by vaccination and enforcement of biosecurity measures.[94] Although the Pakistani H7N3 viruses had so far some capacity for replication in mice and ferrets,

their poor transmissibility suggests that they pose less risk of pandemic potential in comparison to other H7 viruses.[89] The Pakistani H7N3 viruses are closely related to AIV from Europe, Asia, and Australia. Between 1998 and 2000, isolation of LPAIV H7N3 from commercial chickens has been sporadically reported. Therefore, it has been suggested that the introduction of the H7N3 virus occurred in 1994 followed by undetected virus circulation in unknown reservoirs until 2003, possibly in unvaccinated backyard poultry, which have an epidemiological link with the commercial poultry sectors.[91] Although the Pakistani H7N3 lineage diversified into two genetic clusters (1995 and 2003), their antigenicity remained highly conserved[89,95] which could be explained by the failure of those viruses to transmit to other hosts[89] or due to rapid eradication of the virus before emergence of dominant vaccine-escape lineage(s).[95] Genetic analysis showed that HPAIV H7N3 originated from mutation of the progenitor LPAIV (PE**I**PKG**R**/GLF) by insertion of multiple basic amino acids PETPK**RRK**R/GLF in the PCS.[90,91] The Pakistani HPAIV H7N3 had a full length NA stalk. Unfortunately, a full genome sequence of the LPAIV H7N3 reported in April–October 2003 is not available.[91]

Epidemic of H7N3 in Chile

In April 2002, an LPAIV H7N3 with the monobasic PKTR/GLF cleavage motif was isolated from broiler-breeder chickens. One month later HPAIV emerged.[96] Both viruses co-circulated for some period in the index premise.[97] Insertion of ten amino acids (PKT**CSPLSRCRET**R/GLF) in the HA cleavage site of HPAIV was attributed to recombination between the NP and HA genes.[98] Thereafter, viruses with a PKT**CSPLSRCRKT**R/GLF PCS motif evolved.[98] Although both variants of the PCS did not comply with the minimum PCS motif (R-X-R/K-R) of HPAIV,[23,98] the viruses grew well in tissue culture with or without addition of trypsin, whereas the LPAIV grew only in presence of trypsin.[98] Furthermore, a strong tropism of HPAIV H7N3 for the cardiovascular system was demonstrated[99] resembling other HPAIV. In contrast to other LPAIV, the Chilean LPAIV H7N3 replicated also in the cardiac myocytes after intravenous inoculation, which could support that this virus was in the transition stage from low to high pathogenicity.[99] Phylogenetic analysis indicated that NP and PA were most closely related to H7N7 equine influenza viruses but other gene segments were closely related to the North American H7 lineage.[98]

Epidemic of H7N3 in British Columbia (BC), Canada

LPAIV H7N3 was reported from the Fraser Valley (BC) in 52-week-old broiler–breeder chickens on 6 February 2004. The virus was related to LPAIV A/TU/Ontario/2000 (H7N3) which suggested that the latter may have been silently circulated among poultry species and was not detected due to limited active surveillance.[100] Suddenly, an HPAIV H7N3 outbreak was reported in a nearby 24-week-old chicken flock on 17 February.[101] The virus spread to 40 commercial poultry facilities before massive depopulation efforts resulted in complete eradication on 21 May

2004.[100,101] HPAIV H7N3 was isolated from 2 workers 3 days after direct exposure to the infected poultry, both involved in the depopulation of infected birds, suffered from conjunctivitis, headache, and coryza.[101] Increased affinity of this Canadian HPAIV H7N3 toward α2-6 SA linkage predominant in human tracheal epithelial cells was reported.[102] The virus was also capable of using the eye as a portal of entry to mount a productive and lethal infection in the mouse model.[103]

Sequence analysis of the HA genes of both low and high pathogenicity viruses isolated from the index farm, as well as from the 2 infected workers, revealed an insertion of 21 nt at the HA cleavage site of the HPAIV. Non-homologous recombination between the HA gene and seven amino acids of the M1 gene of the same virus was the most probable scenario for the sudden emergence of this HPAIV.[104] The original PCS of LPAIV was PKTR/GLF, while insertion resulted in alteration to PK**QAYQKRM**TR/GLF which subsequently mutated to different forms including PK**QAYRKRM**TR/GLF or PK**QAYKKRM**TR/GLF, PK**QAYHKRM**TR/GLF, PK**QAHQKRM**TR/GLF, PK**QACQKRM**TR/GLF and PK**QAYQKQM**TR/GLF without adverse effect on the pathogenicity.[104,105] The insertion of seven amino acid in the PCS formed an enlarged exposed loop in the HA$_0$ protein which likely enhanced the accessibility of furin-like proteases to the cleavage site and consequently increased the pathogenicity of those viruses.[104,105] No deletion within the NA-stalk domain was observed.

Epidemic of H7N3 in Saskatchewan (SK), Canada

In September 2007, 55-week-old broiler–breeders were found with high H7 antibody titers in sera without showing clinical signs indicating exposure to LPAIV H7N3. A few days later, HPAIV H7N3 emerged. The isolated HPAIV had an insertion of six amino acids within the PCS, PKT**TKPRPR**R/GLF.[106,107] The potential origin of this TKPRPR insert has been assumed to be a recombination with a hypothetical eukaryotic protein of domestic chicken *Gallus gallus*. This limited outbreak was successfully contained quickly without evidence of further spillover to nearby birds.[108] The HPAIV had close phylogenetic relationship with other contemporary LPAIV of wild waterfowl origin in North America suggesting a possible evolutionary relationship.[106] Sequence of this virus revealed full NS1 (230 amino acids), full PB1-F2 (90 amino acids), and no deletion within the NA-stalk domain.

Epidemic of H7N3 in Mexico

On 13 June 2012, several outbreaks caused by HPAIV H7N3 in commercial layer chicken farms in Jalisco, Mexico were reported,[109] resulting in the culling of 4.5 million birds.[110] Until now, the origin of the virus is unknown. However, wild birds and/or poultry trade were suspected to be the source of infection.[110] Vaccination in addition to enforcement of biosecurity measures and test-and-slaughter strategy were statutory control measures.[110] Two farm workers acquired the infection through direct contact with infected chickens. Patients suffered from conjunctivitis without showing any other symptoms and both recovered.[111,112]

Genetically the Mexican H7N3 isolates from poultry and humans are closely related to each other. Apart from the cleavage site, they showed high similarity to LPAI H7N7 viruses in the North American lineage recently isolated from wild birds.[112] The closest H7N3 viruses were chicken and human isolates from Canada.[110] The virus had an IVPI of 2.9. Analysis of the HA indicated an insertion of eight amino acids in the cleavage site PK**DRKSRHRR**TR/GLF which presumably occurred due to recombination of low virulent precursor with a part of the chicken genome. No deletion has been observed in the NA, PB1-F2, or NS1 genes.

Epidemic of H7N7 in the Netherlands

From February to May 2003, a devastating HPAIV H7N7 epidemic occurred in poultry in the Netherlands which also spread to Germany and Belgium.[113-115] Serological screening of Dutch poultry suggested that the HPAI H7N7 epidemic was not preceded by circulation of an LPAIV H7N7 but occurred by reassortment after two separate introductions of AIV from wild mallard into commercial poultry sectors was the likely scenario.[113] It has been stated that the HPAIV A/Chicken/Netherlands/1/2003 (H7N7) acquired the HA gene from A/Mallard/Netherlands/12/2000 (H7N3) and the NA gene from the A/Mallard/Netherlands/2/2000 (H10N7).[113,115,116] The precursor LPAI mallard virus had the monobasic PCS "PK**G**R/GLF", while the HPAIV H7N7 had the multibasic PCS motif "PK**RRR**R/GLF"[115] and no deletion in the NA-stalk region.

Unexpectedly, HPAIV H7N7 infected 89 humans in the largest documented AIV-related outbreak.[117] The virus infected 86 poultry workers and was further transmitted to three of their family members.[117] Although prophylactic use of oseltamivir reduced virus transmission to and between professionals exposed to infected poultry as shown by decreased seroprevalence of H7 antibodies in those persons,[118] a veterinarian died of pneumonia due to handling of infected poultry.[114,119,120] The majority of infected humans suffered from conjunctivitis rather than respiratory illness,[117,119,121] and H7N7 was mostly detected from eye swabs compared with throat swabs.[119] Interestingly, serological surveillance indicated that at least one thousand people contracted H7N7 virus, most of them with no symptoms. It was suggested that the virus transmitted from the primary cases (i.e., cullers, workers, veterinarians, etc.) to approximately half of their household contacts.[121] Interestingly, Belser and associates[102] found that the H7N7 virus retained binding-preferences for the avian α2-3 SA receptor.[122]

Genome sequence of the index HPAIV H7N7 isolated from poultry A/Chicken/Netherlands/1/03 was almost identical to the virus isolated from human A/Netherlands/33/03 who suffered from conjunctivitis; only two silent mutations in the PB2 gene and one amino acid substitution in NS1 protein were uncovered.[115] Furthermore, virus isolates obtained from a family cluster, the wife and daughter of a poultry worker, had only one silent

mutation in the HA gene.[115] However, the genome of the virus isolated from the fatal case had novel 12 synonymous and 14 non-synonymous substitutions in the PB2, NA, HA, PA, and NS1 that did not exist in the virus from the conjunctivitis case nor the index virus isolated from poultry.[115,120] However, these mutations, except PB2 E627K and HA K416R, were also found in viruses from other poultry houses including birds in the farm where the veterinarian contracted the fatal infection,[115] which indicate that deadly viruses with efficient replication in mammals could be generated in poultry.[122] The PB2 E627K and HA K416R substitutions potentially resulted from virus adaptation to the new host as evidenced nine days after the onset of symptoms.[115]

Based on reverse genetics and animal models, the HA, PB2, PA, and NA genes increased replication of the fatal case virus in humans[122] and the HA and PB2 genes were responsible for increased pathogenicity.[120] In a mouse model, the E627K substitution in PB2 was the main determinant of pathogenicity,[120,123] while A143T in the HA introduced a potential N-linked glycosylation site at position 141 near the RBD increased virus titers in the lower respiratory tract and enhanced dissemination of the virus into different organs.[120,122,123] Likewise, the virus isolated from fatal case was also highly virulent for ferrets but did not transmit to contact ferrets while virus isolated from the conjunctivitis case was less virulent and transmitted to 2 out of 3 contact animals.[103]

Interspecies transmission of the virus to other mammals has been recorded. Pigs kept in the same farm with infected poultry were seropositive for H7 antibodies. However, neither clinical signs nor virus shedding was seen and there was no evidence of pig-to-pig transmission.[114] Moreover, van Riel et al.[124] showed that HPAIV H7N7 isolated from the fatal human case was also able to infect cats experimentally and cause respiratory illness but was unable to spread beyond the respiratory tract.

Other Examples Linking Possible Evolution of HPAIV from LPAIV in Nature

Epidemic of H5N1 in Norfolk, England. In December 1991, 7129 out of 8000 18-week-old turkeys died within 8 days in a commercial farm in Norfolk, England.[125] The outbreak was limited and did not spread due to enforced biosecurity and stamping out policy. Since the farm was close to lakes and rivers, possible wild-bird involvement was assumed for the first introduction.[125] Isolation of two H5N1 viruses displaying variable phenotypic characters from a dead frozen carcass was recorded. The virulent A/Turkey/England/50-92/91 virus isolated from a swab sample of the respiratory tract had an IVPI of 3.0, while the avirulent A/Turkey/England/87-92/91 virus isolated from the brain tissue had an IVPI of 0.0.[24,125,126] Interestingly, the avirulent virus could not be recovered from other organs of the same carcass beyond the brain.[24]

Sequence comparison of the HA protein of both LPAIV and HPAIV revealed the identical PCS PQRKRTR/GLF[24] and three amino acid substitutions, K205E, R348G, and G435V.[126] In addition, seven amino acids alterations have been reported in PB2, PB1, PA, and NP.[126] Interestingly, pathogenicity of the LPAIV

could not be increased either by serial passages in specific pathogen-free (SPF) chicken eggs or by intravenous passages in 6-week-old chickens,[127] but succeeded after intracerebral passage in 1- to 2-day-old SPF chicks.[126] Intriguingly, after the first passage of the LPAIV, mutations of the HPAI pathotype (substitution in the NP was retained) were induced but the virus remained LPAIV, IVPI = 0.4, but after the second passage the IVPI amounted to 1.9.[126] Several amino acid changes had been assumed to explain the difference between both pathotypes; namely K123E in PB2, G656E in PB1, and Y74H in PA, which could influence the folding of the polymerase subunits. Nevertheless, there are, so far, no available in vitro reverse-genetic investigations on virulence determinants of this virus.[126]

H5N1 in Guangdong province, China. During the summer and early fall of 1996, geese in a commercial farm in Guangdong province, China suffered from respiratory disorders and up to 40% mortality. Two different H5N1 pathotypes were isolated from this outbreak: A/Goose/Guangdong/1/96 displayed a high pathogenic phenotype for chickens with an IVPI of 2.1 while A/Goose/Guangdong/2/96 replicated poorly in chickens with an IVPI of 0.0.[128-131] Furthermore, the avirulent virus was also detected in several organs (except brain) of experimentally infected geese.[128] The avirulent A/Goose/Guangdong/2/1996 virus was assumed to be the ancestor of the virulent virus and the asymptomatically infected goose were considered to be the ideal reservoir host for spread of the virus to chickens.[128]

Sequence comparison of those two viruses revealed identical multiple basic PCS motif PQRERRRKKR/GLF and only eight nucleotide differences; three synonymous mutations in PB1 and NP and five non-synonymous mutations in PA, NP, M1, and NS1.[128] Using reverse genetics Li and colleagues[128] found that the NS1 gene modulated the virulence of the two viruses in chickens. Thus, the V149A substitution of the NS1 protein was responsible for the difference in virulence observed in chickens since it was able to diminish the ability of the NS1 protein to antagonize the induction of IFN-α and β in chicken embryo fibroblast cells and thus increase virulence and lethality. In geese, it was postulated that NS1 did not play a role in the replication and variation but changes in the NP may be responsible for that.[128] It is worth pointing out that A/Goose/Guangdong/1/96 was probably the immediate precursor of the Asian lineage "Z genotype" of HPAIV H5N1 that spread globally since 2003 resulted in destruction of tens of billions of birds and killed 375 out of 630 (~59.5% fatality rate) infected humans as of 4 June 2013.[131-133] More details on the virulence determinants of the contemporary HPAIV H5N1 in poultry and humans have been previously published.[134-140]

H5N9 in Wisconsin, USA. In February 1968, an outbreak of A/Turkey/Wisconsin/68 (H5N9) caused severe losses in turkeys in Wisconsin, USA.[141] Coexistence of two closely related competitive and distinct virus populations within the original virus isolate was reported.[142,143] The virulent strain induced rapid embryo lethality of SPF chicken embryonated eggs and mortality in four-week-old chicks while the avirulent pathotype induced slowly embryo lethality (more than 72 h) and was nonlethal to chicks. Both virus populations were stable through 12 serial passages in SPF eggs.[144] Two amino acid differences in the NS gene were

found:[145] valine and phenylalanine (in the nonlethal phenotype) or leucine and serine (in the virulent phenotype) at position 119 in NS1 and 48 in NS2, respectively. Nevertheless, these mutations did not affect the rates of viral replication neither in cell culture nor in embryonated chicken eggs.[145] The avirulent strain had monobasic PCS PQRETR/GLF.[146] However, so far no genomic data are available for the virulent virus. Therefore, further investigation is required to determine the virulence markers of those two viral populations mentioned in the earlier studies.[142-145]

Epidemic of H7N7 in Victoria, Australia. In January 1976, a limited outbreak of AIV occurred on a combined layer and broiler farm in Victoria, Australia.[147] Recorded mortality was up to 25%[148] and the disease was eradicated by culling.[149] The source of the virus was not traced.[148] Isolation of H7N7 viruses displaying different virulence was recorded. A/Chicken/Victoria/76 caused classical AIV signs with variable pathogenicity indices ranging from 0.97 to 1.98,[149-152] whereas A/Duck/Victoria/76 isolated from asymptomatic commercial ducks had an IVPI of 0.00 and induced, contrary to the chicken isolate, no clinical signs in experimentally infected chickens, turkeys, and ducks.[150,152] Cleavage site of the virulent chicken virus specified P**KKREKR**/GLF while the avirulent duck virus had P**KKR**/GLF.[149,153] Although the two viruses showed different pathogenicity and the chicken virus was isolated first,[152] it remains speculative which virus altered its phenotype.

General Remarks and Outlook

Molecular changes found in the naturally evolving HPAIV are thought to be a major contributing factor for the appearance of devastating epidemics besides ecological niches, the host's immune system, the intracellular machinery of the host and their complexity. From this comprehensive review, it could be concluded that: (1) wild birds were confirmed or claimed as a source of first introduction of LPAIV to the terrestrial poultry. (2) The elapsed time between first introduction of LPAIV and emergence of HPAIV in poultry is extremely variable from few days to several months and even years. (3) Molecular details of evolution of highly virulent viruses from LP precursor(s) seem to be virus-dependent. (4) Acquisition of cleavage site alterations occurred due to insertion of basic amino acids or conformational changes that make the PCS more accessible to ubiquitous proteases. (5) Existence of multibasic amino acids in the PCS was not the sole genetic change; concurrent mutations in the HA adjacent to or apart from the PCS were also observed. (6) Compatible genetic constellation to generate a virulent virus was essential, hence some viruses (from Mexico, Canada, Chile, Pennsylvania, and Italy) required only few point mutations while others (from Texas, the Netherlands, and Pakistan) probably evolved by a multistep process of reassortment and mutations. (7) Several changes have been observed in all gene segments particularly the polymerase subunits, in particular the PA gene (**Table 1**). The NEP had very few, if any, differences between both pathotypes which may exclude any direct role in shift of pathogenicity of LPAIV. (8) Deletion of the NA stalk region was observed in all H5N2 outbreaks. In contrast, only the Italian H7N1 had a 22 amino acid deletion while neither H7N3 nor N7N7 subtypes specified an NA-deletion[154] indicating that this deletion is NA-subtype-specific. Whether internal gene segments can influence this HA-NA meticulous balance needs further investigation. (9) Extensive pangenomic non-synonymous mutations were observed in viruses isolated from all outbreaks and their role in pathogenicity or adaptation has not been elucidated yet. (10) Evolution of influenza viruses by recombination has been previously reported,[155-160] but in this review it has been recorded in four outbreaks which should be a hot-spot for future research. (11) Some viruses crossed species barriers and infected a wide host spectrum while others circulate among much narrower range of hosts (i.e., Pakistani H7N3 viruses). (12) Effective control of these outbreaks was successful mainly by elimination of the infected birds and sometimes with adoption of inactivated vaccines developed from local LPAI strains (i.e., Mexico and Pakistan). (13) Genetic markers mentioned here should be considered in studies of virulence, pathogenesis, adaptation and transmissibility of AIV using reverse genetics. Although data in this area of research is substantially growing, different facets of virulence determination exist and further investigation is highly required. Our increased understanding of the genetic changes accompanying the shift from LPAIV to HPAIV under natural conditions may help to assess the risk posed by any LPAIV after introduction into domestic poultry to become HPAIV. It may also assist in the development of new attenuated vaccines specifically targeted at the most relevant LPAIV circulating in nature.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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