Review

The changing nature of avian influenza A virus (H5N1)

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Highly pathogenic avian influenza A virus subtype H5N1 has been endemic in some bird species since its emergence in 1996 and its ecology, genetics and antigenic properties have continued to evolve. This has allowed diverse virus strains to emerge in endemic areas with altered receptor specificity, including a new H5 sublineage with enhanced binding affinity to the human-type receptor. The pandemic potential of H5N1 viruses is alarming and may be increasing. We review here the complex dynamics and changing nature of the H5N1 virus that may contribute to the emergence of pandemic strains.

Influenza A host range

Influenza A viruses are categorized into subtypes based on the antigenic properties of their two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA subtypes and nine NA subtypes have been identified. All 16 HA and nine NA subtypes are maintained in influenza A viruses in aquatic bird populations, especially ducks, shorebirds and gulls [1,2]. Wild waterfowl are the major reservoir of influenza A viruses and infected hosts are usually asymptomatic, indicating a long-standing equilibrium [1]. The virus has an extremely wide host range, from birds to various mammalian species including humans, pigs, horses and dogs [1,3]. We describe here the ecology and dynamics of avian influenza (AI) viruses, review the latest findings on the changing nature of highly pathogenic AI (HPAI) viruses subtype H5N1 in nature, focusing on their HA receptor-binding specificities, and discuss their pandemic potential and the strategy for disease control.

Viral transmission of influenza

AI viruses in poultry cause a variety of clinical diseases and are classified on the basis of their virulence in chickens [4]: HPAI viruses cause systemic lethal infections with mortality of up to 100% within 48 h post-infection [5], whereas low pathogenic AI (LPAI) viruses, which are the majority of AI viruses, cause outbreaks with lower mortality and rarely generate outbreaks of severe disease in poultry [1]. HPAI viruses have been reported to emerge after transmission of LPAI virus subtypes H5 and H7 from wild reservoirs to gallinaceous birds and subsequent selection of mutant viruses [3,4]. LPAI viruses replicate in intestinal and respiratory organs and are shed mainly in the feces of infected birds, with some oropharyngeal shedding [6]. Thus, virus transmission occurs primarily by the fecal–oral route [1]. By contrast, HPAI viruses replicate systemically in poultry and are more readily transmitted by nasal and oral routes [2].

AI viruses have been isolated from various animals other than aquatic birds, including humans, pigs, horses, dogs, cats and terrestrial birds, indicative of their interspecies transmissibility [1,3,7]. However, most AI virus transfers to primate species have resulted in limited spread, with infection confined to the initial recipient and a restricted number of close contacts [3,8]. The viruses replicate primarily in the upper respiratory organs of these hosts, causing respiratory disease [1]. Differences in viral tissue specificity are the result of biological differences between influenza virus reservoir hosts (donor hosts) and recipient hosts [9]. Various host factors that affect the viral life cycle have been identified including cell-surface receptors [10,11], intracellular environment [12,13], body temperature [14] and immune responses [15]. Thus, if a virus infects and adapts to a recipient host, the virus accumulates nucleotide sequence changes in different genes at different rates, leading to evolution of a species-specific virus lineage [8].

Evolution of influenza A viruses

Influenza A viruses contain segmented single-stranded RNA (ssRNA) and are continuously evolving. Small base changes occur by point mutations, known as genetic drift, which is driven by the infidelity of the virus-encoded polymerase [7,16]. Major changes occur less frequently by reassortment of genes between two different virus strains in coinfected cells, leading to a genetic shift [3,7]. Genetic reassortment can result in the emergence of novel subtypes with completely new antigens [17]. Through these genetic processes, influenza viruses acquire the ability to sidestep host immune responses continually, causing significant seasonal disease and sporadic pandemics.

Pandemic influenza has occurred four times in the last century: in 1918 ('Spanish' flu, H1N1), 1957 ('Asian' flu, H2N2), 1968 ('Hong Kong' flu, H3N2), and 1977 ('Russian' flu, H1N1) (Figure 1). The data suggest that the 1918 pandemic was caused by an AI virus that had infected

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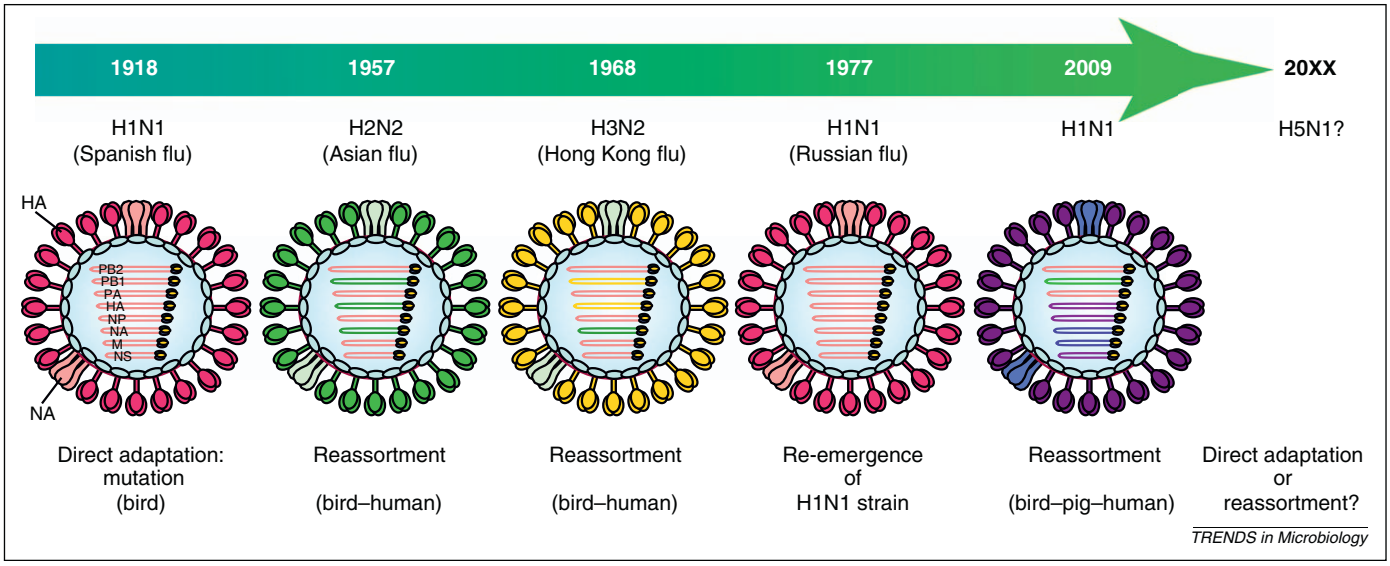


Figure 1. Timeline and evolution of pandemic influenza viruses. Pandemics in the 20th century were caused by infection by an avian influenza virus (1918), an avian–human reassortment virus containing avian HA, NA and PB1 (1957), and an avian–human reassortment virus containing avian HA and PB1 (1968), with the other gene segments from the circulating human virus. Re-emergence of the H1N1 strain that circulated in the 1950s led to a limited pandemic in 1977. The latest pandemic influenza (2009) was caused by an avian–swine–human reassortment producing a virus containing PB2 and PA from an avian virus, PB1 from a human virus, and the other gene segments from two distinct lineages of swine viruses. Future pandemic strains could arise through any of the mechanisms: adaptation by accumulation of mutation(s), and genetic reassortment and re-emergence of a virus that has not infected humans for a generation. Common names of the past pandemics and the virus origins are shown in parentheses at the top and bottom, respectively.

and adapted to replication in humans, the 1957 and 1968 pandemics by reassortments producing human influenza viruses containing HA and PB1 gene (and/or NA gene) segments from an avian virus, and the 1977 pandemic by re-emergence of the H1N1 virus [2,18]. However, the most recent influenza pandemic emerged in 2009 by an avian–swine–human reassortment producing a virus containing PB2 and PA genes from an avian virus, PB1 from a human virus, and the other gene segments from two distinct lineages of swine viruses [7,19].

Aside from the past human pandemics, five AI subtypes are known to have produced sporadic human disease after direct transmission from an avian host: HPAI viruses H5N1, H7N3 and H7N7, and LPAI viruses H7N2, H7N3, H7N7, H9N2 and H10N7 [3]. The mechanism(s) that determine the transmissibility to humans and pandemic potential of these AI viruses have still not been fully elucidated [7,18]. Three criteria for a new influenza virus strain to have the potential to produce a pandemic are: (i) emergence (or re-emergence) of an influenza virus HA subtype that has not infected humans for at least one generation, (ii) high infectivity in humans, and (iii) sustainable transmission among humans (Figure 2a) [3,9]. However, because viral adaptation in different hosts is multifactorial [9], no clear genetic pattern has emerged to define the changes necessary for the evolution of a pandemic human virus [7]. It is uncertain which route the next pandemic influenza virus will take (Figure 1).

The HPAI H5N1 virus is not currently a pandemic virus and remains an avian virus [20,21], but it is undergoing continual evolution [22]. Outbreaks of HPAI H5N1 viruses were first recorded in poultry in Guangdong, China in 1996 [23]. Since its emergence the A/goose/Guangdong/1/96 (Gs/GD) virus lineage has probably become the best-studied HPAI virus (Figure 3, upper panel). This virus has gradually become endemic in poultry in different regions of China, developing into genetically and antigenically distinct sublineages.

Influenza virus surveillance in China has revealed that since 1997, the Gs/GD virus lineage has undergone frequent reassortment with different AI viruses that were circulating in the region and has generated many different viruses with a genetic shift (or genotype) [2,24]. These reassortment viruses had considerable variability in their combination of internal genes, although each had a Gs/GD virus-derived HA. Based on their internal genes, the genotypes were designated A, B, C, D, E, V, W, X0-3, Y, Z and Z+ [2,24]. Furthermore, a novel genotype, designated P, emerged in the Lao People's Democratic Republic (PDR) in 2007, marking the first case of reassortment between the Gs/GD virus lineage and another AI virus outside China [25]. Continuing outbreaks of the Gs/GD virus lineage have also led to the accumulation of point mutations in the viral HAs, generating genetic and antigenic changes. According to the current classification, HA genes are grouped phylogenetically into clades 0–10 [4]. These HA clades correlate well with antigenic differences [4]. Thus, reassortment of Gs/GD internal genes and antigenic evolution of its HA by point mutations have been interactively involved in the emergence of dominant variants of the Gs/GD virus lineage [26].

The ecology of the Gs/GD virus lineage has varied over time [4,27]. Historically, HPAI has emerged transiently, mainly in localized areas in populations of gallinaceous species, owing to mass die-offs due to the high mortality of HPAI infections (Figure 4a) [28]. HPAI viruses were also thought not to persist in wild bird populations [28]. In fact, there had been little evidence of Gs/GD virus lineage infections in wild birds, even during the early endemic infections in land poultry in 1996–2001 (Figure 4b) [4]. However, this situation has changed dramatically since 2002 because aquatic and terrestrial wild birds died following infection

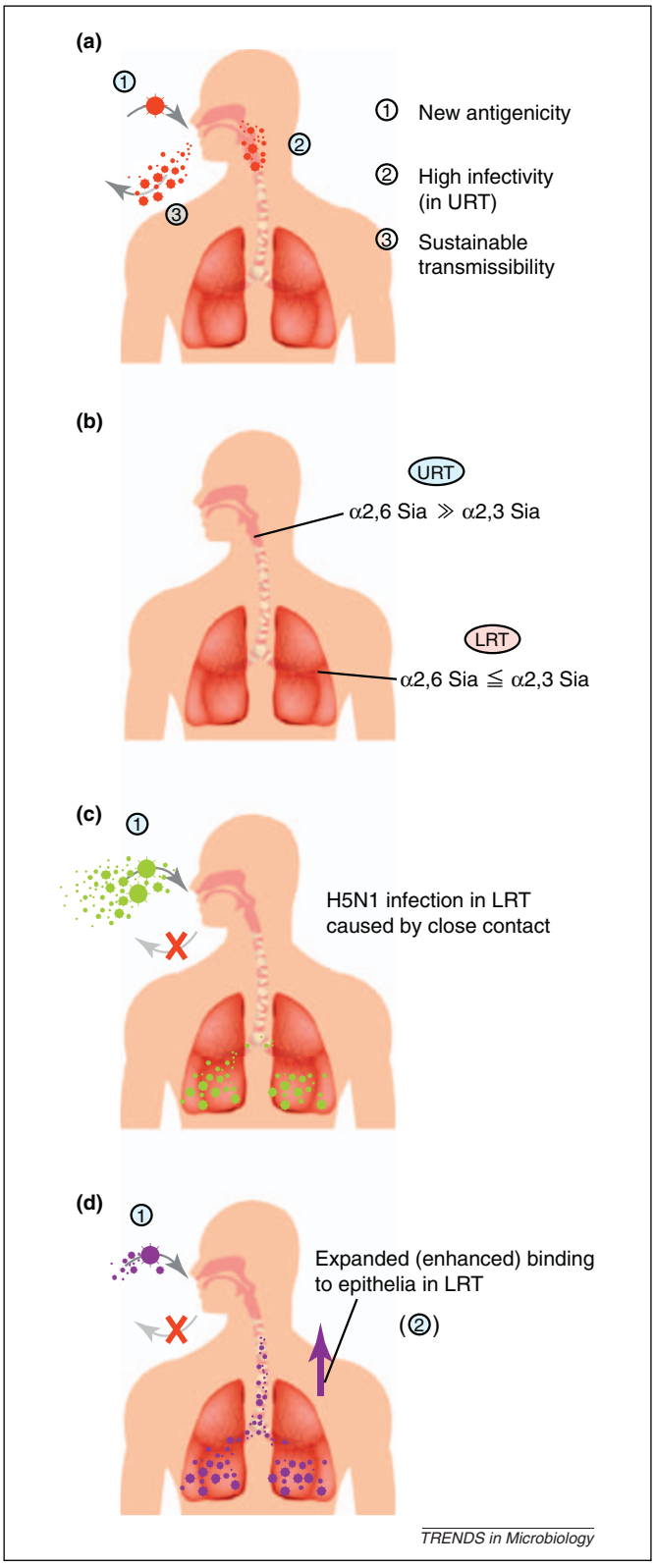


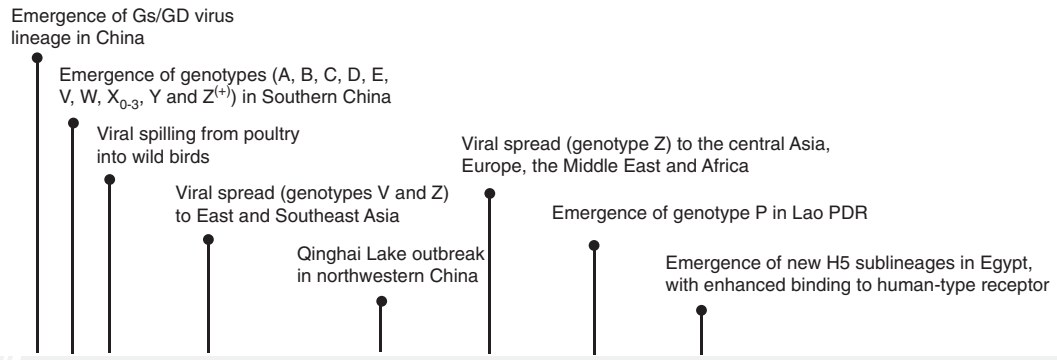
Figure 2. Influenza virus adaptation in humans for a pandemic. **(a)** Criteria for influenza virus to be a pandemic strain. The three criteria shown here must be met for a new pandemic to arise. URT, upper respiratory tract. **(b)** Distribution of α2,3 and α2,6 sialylglycans in the human respiratory tract. The URT contains abundant α2,6 sialylglycan (α2,6 Sia; human-type receptor) and less α2,3 sialylglycan (α2,3 Sia; avian-type receptor). By contrast, the lower respiratory tract (LRT) contains substantial α2,3 Sia. This distribution pattern imposes a primary species barrier for AI viruses. **(c)** Pathology of the Gs/GD virus lineage (H5N1) in humans. The virus lineage only has binding affinity for α2,3 sialylglycan and needs to reach the LRT [52,53]. The pandemic criterion met is marked as a circled number. **(d)** Pathology of the H5 sublineage of the Gs/GD virus lineage that has emerged in Egypt. Expansion of receptor usage (increased α2,6 sialylglycan binding) enables the new sublineage to bind more efficiently to epithelia in the LRT than the parental virus lineage, but this new sublineage still lacks binding affinity to the URT and sustainable transmissibility in humans.

with Gs/GD lineage viruses (Figure 4c) [1,4]. Large outbreaks of genotype Z viruses at Qinghai Lake in northwestern China in 2005 characterized the strikingly varied phenotypes of this virus lineage, with high mortality in a variety of wild aquatic birds [24,29]. The viruses then spread unexpectedly over large geographic areas, apparently via wild bird migration in winter 2006, spreading westward to Central Asia, Europe, the Middle East and Africa [30]. Several experimental infection studies also suggested a role of wild birds in the spread and maintenance of recent Gs/GD lineage viruses [6,31]. As of August 2011, 7030 poultry and/or wild-bird outbreaks have been reported from over 50 countries and HPAI has become endemic in birds in at least four countries (China, Indonesia, Vietnam and Egypt) (World Organization for Animal Health; http://www.oie.int/animal-health-in-the-world/update-on-avian-influenza/). Although large outbreaks in wild birds have rarely been reported since 2006, the virus appears to have been maintained in wild bird populations, providing opportunities for spread back to domestic poultry that make it difficult to control. Such intertwined ecology of the Gs/GD virus lineage has recently generated multiple H5 sublineages in endemic areas. Among them, a number of phylogenetically and antigenically distinct H5 sublineages have been isolated in Egypt, but none has yet become dominant, and these viruses are currently co-circulating in the local bird population [32]. Co-circulation of different H5 sublineages has also allowed reassortment among the sublineages in Vietnam [33] and Nigeria [34]. Therefore, the Gs/GD virus lineage has reached epizootic levels in both domestic and wild bird populations across Eurasia and Africa.

Compared to earlier H5N1 viruses, the recent Gs/GD virus lineage has shown the ability to cross the species barrier and infect a broad range of mammalian species, including humans [32,35–37]. The cumulative number of confirmed human cases of H5N1 infection reported to the World Health Organization (WHO; http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/) to date is 566 with 60% mortality (Figure 3, lower panel). More than 80% of the total human H5N1 influenza cases have been reported in AI endemic areas, indicating hotspots for bird-to-human transmission. In addition to infections in humans, other mammals such as tigers, leopards, dogs, cats and a stone marten have recently been symptomatically infected with an HPAI H5N1 virus by feeding on bird carcasses [27]. Palm civets [35], donkeys [36] and pigs [38] were also shown to be naturally infected with this virus lineage. Furthermore, experimental infections have been established in mice, ferrets, monkeys, cattle and foxes [27]. Thus, the current Gs/GD virus lineage has exceptionally strong zoonotic properties.

Receptor binding

Influenza virus infection requires binding of viral HA to host glycans or gangliosides that terminate in sialic acids (Sias), but there are distinct differences in the forms of



Country	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths	Cases	Deaths
China	1	1	0	0	8	5	13	8	5	3	4	4	7	4	2	1	0	0	40	26
Vietnam	3	3	29	20	61	19	0	0	8	5	6	5	5	5	7	2	0	0	119	59
Thailand	0	0	17	12	5	2	3	3	0	0	0	0	0	0	0	0	0	0	25	17
Cambodia	0	0	0	0	4	4	2	2	1	1	1	0	1	0	1	1	8	8	18	16
Indonesia	0	0	0	0	20	13	55	45	42	37	24	20	21	19	9	7	8	6	179	147
Azerbaijan	0	0	0	0	0	0	8	5	0	0	0	0	0	0	0	0	0	0	8	5
Turkey	0	0	0	0	0	0	12	4	0	0	0	0	0	0	0	0	0	0	12	4
Egypt	0	0	0	0	0	0	18	10	25	9	8	4	39	4	29	13	32	12	151	52
Nigeria	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1
Other	0	0	0	0	0	0	4	2	6	3	1	0	0	0	0	0	2	0	13	5
Total	4	4	46	32	98	43	115	79	88	59	45	33	73	32	48	24	50	26	566	332

From WHO, updated on 10th October 2011.

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Figure 3. Timeline of Gs/GD virus lineage epidemiology. (Upper panel) Major events since 1996 in the Gs/GD virus lineage (H5N1), which is epizootic in bird species. (Lower panel) Confirmed human H5N1 infection cases reported to the World Health Organization (WHO; http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/). It is noteworthy that emergence of a new H5 sublineage in Egypt, with enhanced binding to human-type receptors, caused that country to have the highest number of human infections worldwide after 2009.

these molecules that are recognized (Figure 5a) [10,39]. Virus affinity for different sialyl sugar structures is an important determinant of virus host range and pathogenicity [2,10].

Sias are a family of sugars with a nine-carbon backbone that are typically found attached to the end of glycoconjugate chains [Figure 5a(i)] [10,39]. There are over 50 natural modifications of the Sia core structure, including esterification with acetyl, glycolyl, lactyl, sulfate and phosphate groups [39]. Sias bind to cell-membrane sugars through an α2,3, α2,6, α2,8 or α2,9 linkage catalyzed by sialyltransferases that are expressed in a tissue- and species-specific manner [Figure 5a(ii)] [40]. The most common sialyl terminal substituents are *N*-acetylneuraminic acid-α2,3-galactose (NeuAcα2,3 Gal) and *N*-acetylneuraminic acid-α2,6-galactose (NeuAcα2,6 Gal) [10]. Human influenza viruses preferentially bind to sugar chains ending in NeuAcα2,6 Gal, whereas most avian viruses preferentially bind to sugar chains ending in NeuAcα2,3 Gal [41,42]. As expected from this specificity, human upper-airway epithelia express mostly NeuAcα2,6 Gal [43], whereas duck intestinal epithelia express mostly NeuAcα2,3 Gal [41,44]. This provides an interspecies barrier preventing avian viruses from easily infecting humans. However, swine tracheal epithelial cells contain both NeuAcα2,3 Gal and NeuAcα2,6 Gal [45,46], which explains why pigs are highly susceptible to both human and avian viruses and serve as an intermediate host acting as a 'mixing vessel' [47].

Surprisingly, it was recently found that this restriction mechanism(s) is not a complete barrier to interspecies transmission [48]. An HPAI H5N1 virus that only binds to an α2,3 Sia linkage emerged in China in 1997 and has been occasionally been transmitted directly from birds to humans [20,49]. Recent lectin histochemistry analyses demonstrated that the NeuAcα2,3 Gal receptor for AI viruses is more widely expressed in the human respiratory tract than was previously thought [43]. Although NeuAcα2,6 Gal oligosaccharides are dominant on ciliated and non-ciliated cells in human upper-airway epithelia, NeuAcα2,3 Gal oligosaccharides are also located on ciliated cells [50,51] and alveolar epithelia (Figure 2b) [52,53]. The expression profile of NeuAcα2,6 Gal and NeuAcα2,3 Gal on cell surfaces also changes depending on the disease and host age [11,54,55]. These findings suggest that the potential of direct bird-to-human transmission of avian viruses with a NeuAcα2,3 Gal preference may be greater than previously supposed [56,57].

Chandrasekarn *et al.* recently revised this linkage paradigm by showing that the topology of sialylated pentasaccharides, widely present in the human upper airway, can modulate the receptor-binding properties of influenza A viruses beyond specific α2,3 and α2,6 Sia linkages [Figure 5a(iii)] [51]. Mass spectrometry analyses have shown a remarkable diversity of sialylated *N*-glycans in human upper respiratory epithelia, with long α2,6 oligosaccharide branches (i.e. pentasaccharide or longer) predominating. Although this observation suggests a high

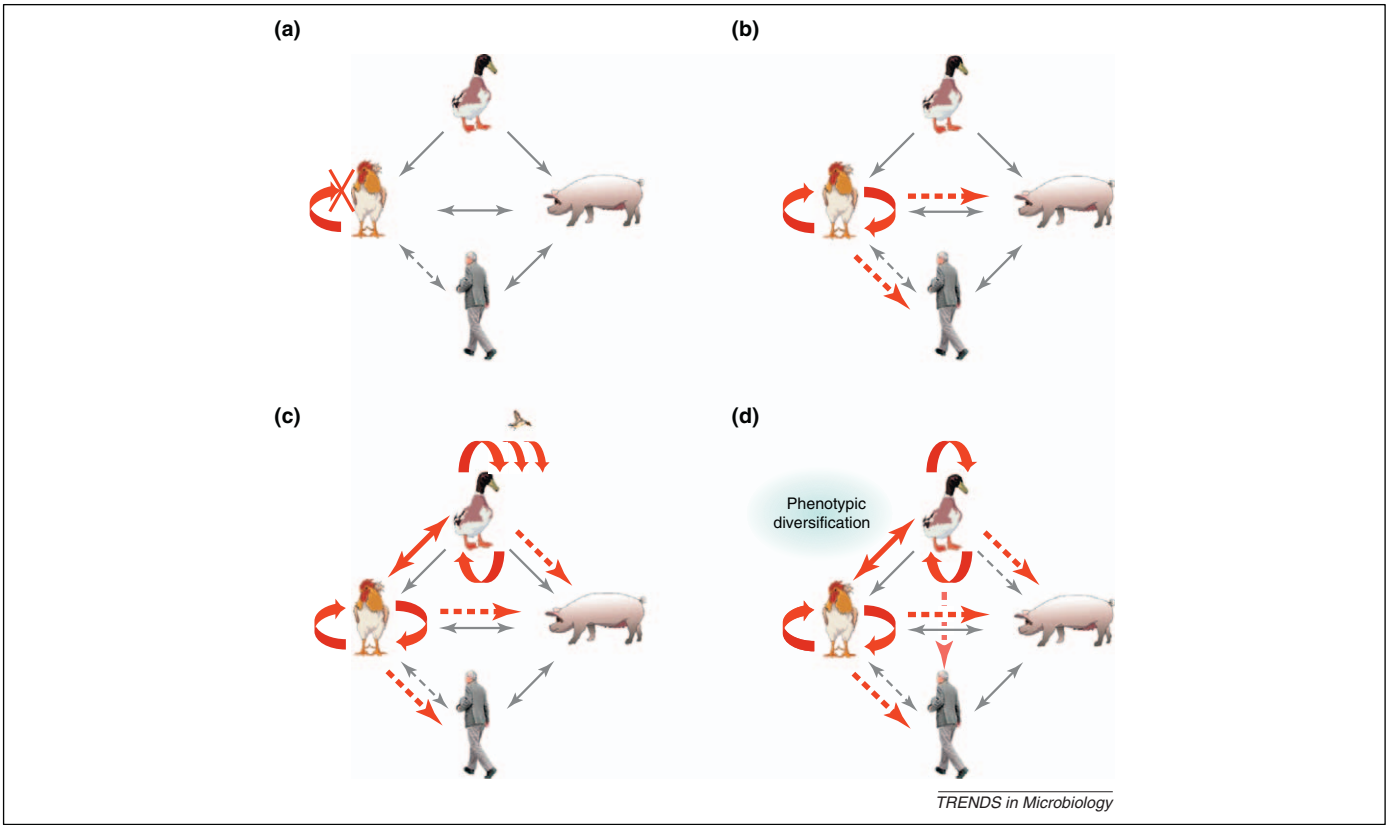

Figure 4. Changing ecology and interspecies transmission of the Gs/GD virus lineage. Schematic of interspecies transmission between wild waterfowl, land-based poultry, pigs and humans. Grey arrows represent the infectious routes of influenza A virus. Red arrows show dissemination of the Gs/GD virus lineage (H5N1). Frequent viral cross-species transmission is depicted as a broken arrow or a dotted arrow. **(a)** Before the emergence of Gs/GD virus in 1996, highly pathogenic avian influenza had emerged transiently in land-based poultry in localized areas (traditional outbreak dynamics). **(b)** In 1997–2001, the Gs/GD virus lineage became endemic in land-based poultry in China and caused sporadic infections in humans and pigs. **(c)** In 2002–2006, several genotypes of the Gs/GD virus lineage infected a variety of wild birds and caused severe outbreaks. The Gs/GD virus lineage unexpectedly spread outside China in 2006 and thereafter spread over large distances through bird migration. **(d)** Since 2007, virus persistence in a wide range of birds has allowed phenotypic diversification in endemic areas and has led to the emergence of a new H5 sublineage in Egypt with enhanced binding affinity to the human lower respiratory tract. Also, a higher risk of human H5N1 infection due to contact with infected waterfowl has been reported recently in Egypt [32]. Thus, these results suggest expanding possibilities for viral transmission from birds to humans.

complexity of HA ligands in the respiratory tract, glycan–HA co-crystal structures showed that long human α2,6 sialylated glycans have a bent topology, whereby even distant carbohydrate groups contribute to HA binding via numerous contacts between carbohydrate residues 1–5 and the HA molecules (Figure 5b, lower panel) [51,58]. However, α2,3 and short α2,6 *N*-glycans invariably form a narrow topology for HA binding, and their interaction with the HA primarily involves contact between amino acids and only the first and second sugars (Figure 5b, upper panel). Indeed, HAs from human-adapted H1N1 and H3N2 viruses, but not from AI viruses, specifically bind to long α2,6 sialylated glycans, whereas only short *N*-glycans are receptors for avian strains regardless of the Sia linkage type [51,59]. These results suggest that recognition of the characteristic topology of long human α2,6 glycans, but not the α2,6 linkage itself, might be crucial for adaptation and pandemic spread of AI viruses in humans.

Influenza viruses recognize the further complexity generated by the sugar composition of glycan internal carbohydrate units [Figure 5a(iii)] [60]. Screening of HAs on a glycan microarray showed that the receptor specificity of different HAs differs markedly not only for Sia linkages but also for other internal glycan modifications, such as sulfation [61]. Furthermore, a study of H5N1 virus infection in *ex vivo* cultures of human nasopharyngeal tissues, on which Siaα2,3 Gal was not detected by a linkage-specific lectin, indicated that current HPAI H5N1 viruses could use other receptors via currently undetermined glycan topology to infect human upper airway epithelia [62]. In summary, it appears that influenza viruses recognize more complex glycan topologies on their target cells, which have not been fully elucidated, and different viruses use a different range of glycan receptors for viral entry [Figure 5a(i)–(v)], although human and avian virus HAs have primary specificity for α2,6- and α2,3-linked sialosides, respectively.

Spread of strains with increased affinity for α2,6 Sia

Almost all H5N1 HPAI viruses isolated from humans thus far display preferential binding for α2,3 Sia (Figure 2b,c) [44]. Several cases have been reported in Asia in which changes in viral amino acid residues surrounding the HA receptor-binding domain appear to have been positively selected in patients infected with HPAI H5N1 viruses [63,64]. These findings indicated that adaptation of this virus lineage to humans can take place by modification of receptor specificity. Indeed, several amino acid substitutions in H5 HAs conferring enhanced binding to α2,6 Sia have been described in virus isolates from patients or have

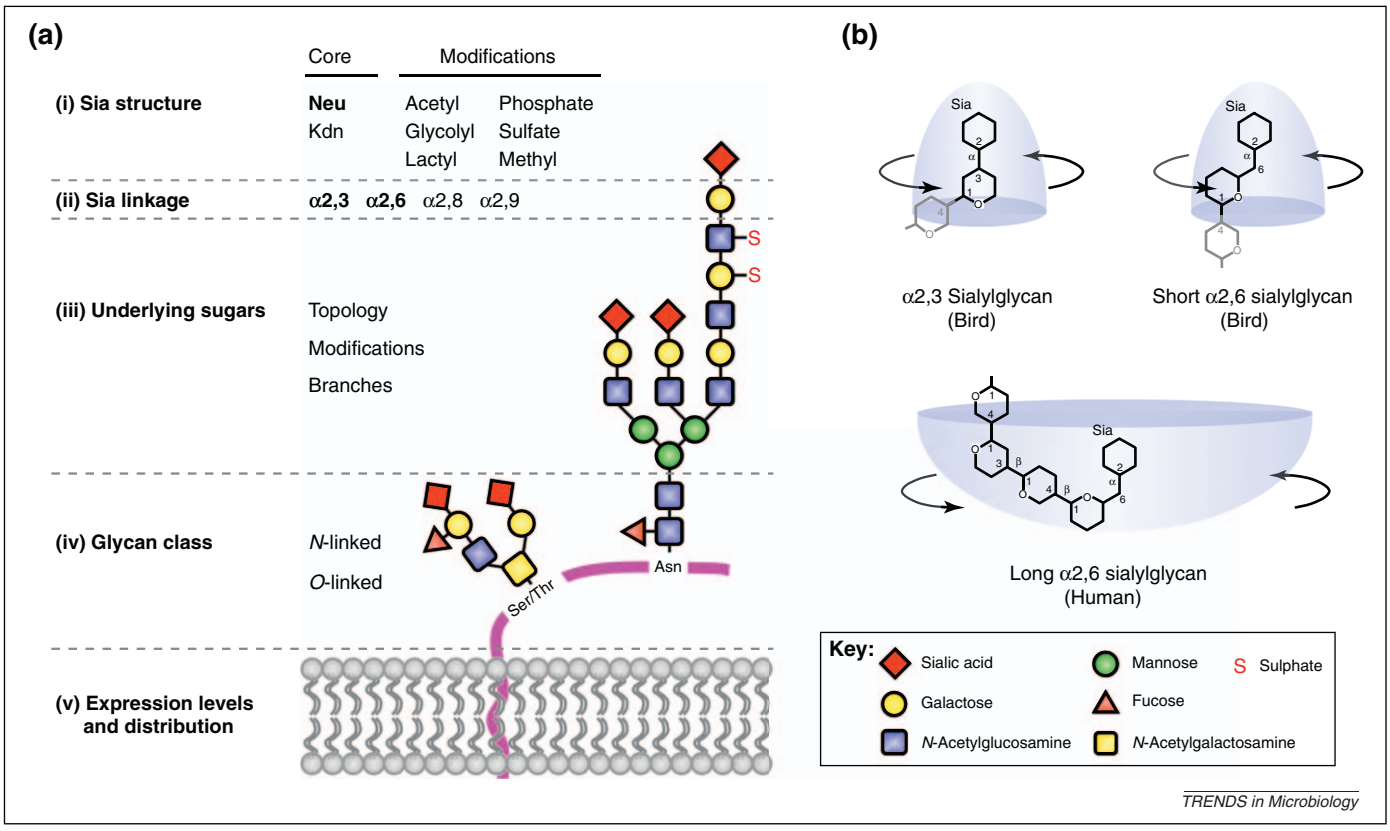

Figure 5. Complexity of sialylglycans recognized by influenza A virus. **(a)** Model of sialylglycan for analysis of hemagglutinin (HA) binding to receptors. **(i)**–**(v)** Representative structures and modifications of the glycans. The structural information in the entire glycan chain influences viral binding specificity, although human and avian virus HAs have a primary specificity for either terminal NeuAcα2,6 Gal or NeuAcα2,3 Gal, respectively (bold). The figure was adapted, with permission, from [39]. **(b)** Sialylglycan structure required for human adaptation of influenza A virus. Distinctly different topologies are adopted by α2,3 and short α2,6 sialylglycans (characteristic in birds) and long α2,6 sialylglycans (characteristic in humans) for binding HA. α2,3 and short α2,6 sialylglycans interact with HA with the first and second sugars in a narrow 3D topology. By contrast, long α2,6 sialylglycans, which are widely present in human upper airway epithelia [e.g. glycans containing the lactosamine repeat $(Gal\beta1,4GlcNAc\beta1,3)_n$)] contact HA via their first to fifth carbohydrate residues in a wide 3D topology. Al viruses need to adapt to the characteristic human long α2,6 sialylglycan topology to infect humans efficiently. The figure was adapted, with permission, from [58].

been introduced experimentally [32,63,65,66]. However, the impact of such modifications on virus transmissibility to humans has not been fully elucidated, and there have been only limited reports of these mutations in H5N1 in human influenza cases [63,64].

By contrast, recent epidemiological studies of H5N1 found diverse populations of H5N1 viruses with altered receptor affinity in birds. In the Lao PDR, the novel genotype P virus emerged in 2007, with reduced affinity for α2,3 Sia [25]. More recently, some new sublineages of genotype Z have emerged in local bird populations in Egypt with enhanced α2,6 Sia receptor affinity and binding affinity for α2,3 Sia [32]. It was noted that these sublineages had increased attachment to and infectivity in the human lower respiratory tract. The emergence of this sublineage in 2008 coincided with an increase in human H5N1 virus infection cases in Egypt, causing this country to have the highest number of human H5N1 cases worldwide since 2009 (Figure 3). These findings suggest that viral acquisition of α2,6 Sia binding during viral diversification in birds can contribute to an increase in bird-to-human transmission efficiency (Figure 4d) [32]. Maintenance of the Gs/GD virus lineage in birds has clearly driven such phenotypic variation in the field, and this should be considered in all countries where H5N1 is endemic [25]. Because H5 variants have emerged and are circulating in some bird populations [32] that have habitats in human rural and urban areas, the emergence of such viral mutations in bird species could be important risk factors for human infections. The mechanism underlying the emergence of H5N1 viruses in Egypt with both α2,3 Sia and α2,6 Sia binding affinity is unclear. Geographic and cultural factors inherent in this region could provide different evolutionary pressures affecting the epidemiology of H5N1 viruses, leading to complex evolution of H5N1 viruses in nature with differing Sia-binding affinities [4].

As described above, some strains of the Gs/GD virus lineage have acquired binding affinity for the α2,6 linkage [32,66], but still have inefficient transmissibility to humans. One explanation for this restriction might be that a complete change in receptor specificity from α2,3 to α2,6 is necessary for virus replication to adapt to the human upper respiratory tract and for efficient viral transmission among humans [27,52]. In fact, most variant H5N1 strains have retained binding affinity for avian-type receptors [32,63,65,66], in contrast to the earliest isolates from the 1918, 1957 and 1968 pandemics, which had binding affinity for NeuAcα2,6 Gal even though their HAs were derived from an avian virus (Figure 2a,b) [67]. Another explanation could be that acquisition of HA binding to long α2,6 sialyl glycans, characteristic of human upper-airway epithelia, is a necessary condition for virus adaptation for human

infections, but loss of the ability to bind to an α2,3 linkage is not necessary for efficient transmission among humans [51,59], as observed for the human H1N1 A/Texas/36/91 strain [68]. The new H5 sublineages in Egypt, with significantly enhanced binding affinity for α2,6-linked sialylglycopolymers, have increased attachment to human tracheal and alveolar epithelia, but undetectable attachment to the larynx (Figure 2d) [32]. This implies that the Egyptian H5 sublineage recognizes different glycan topologies in human upper- and lower-airway epithelia, which is still unexplained. All H5N1 variants with some α2,6 Sia-binding affinity [32,63,66] probably have not yet acquired receptor binding affinity to long human α2,6 glycans. In fact, efficient transmission of the HPAI H5N1 virus has not occurred in humans or in experimental mammalian models of transmission [69,70].

However, the increasing host range for the Gs/GD virus lineage and repeated infections could drive mutations conferring efficient human-to-human transmission [71]. Indeed, Chutinimitkul *et al.* have recently reported that some HA mutations, introduced experimentally, can cause a complete switching of receptor specificity in an Indonesian AI virus [72]. Enhanced binding to α2,6 Sia is an important initial step for adaptation of avian viruses to infect humans [9,53]. Such a step in the Gs/GD virus lineage has probably occurred, at least partially, in Egypt, where new H5 sublineages have expanded their receptor specificity from alveoli to trachea in the human airway, as described above (Figure 2d and 4d) [32]. It may not be necessary for this virus lineage to use an intermediate (mixing vessel) host to form a pandemic virus strain because its increased attachment to human airway eptithelia would enable reassortment between avian and human influenza viruses or adaptation of avian viruses directly in humans. It is also noteworthy that the new H5 sublineage in Egypt was reported, according to bioinformatic analysis, to have evolved toward a Spanish flu virus-like receptor-specificity for human infection [73]. Furthermore, the currently widespread genotype Z viruses, including the Egyptian H5N1 viruses, have viral polymerase PB2 with mammalian-type PB2-627Lys [29], which enhances the host range and virulence of influenza A viruses [14,74–76], implying an increased potential for evolution to a pandemic virus. Thus, the current Gs/GD virus lineage is diversifying (Table 1) in a way that might increase its human pandemic potential. However, it is not known how long it would take for a strain with pandemic potential to acquire human transmissibility and whether the Gs/GD virus lineage could overcome the species barrier in the future (Box 1) by further optimizing its replicative machinery for humans, which is probably needed for overall viral fitness [9]. Finally, it should be noted that not only HA but also other viral gene products (e.g. PB2, PB1-F2, NA and NS1) confer viral adaptation to mammals, interacting with host factors during various steps in viral infection, including replication, nuclear export, assembly, budding, and antagonism of the host antiviral response (reviewed in [2,7,12,13]).

Box 1. Outstanding questions

- Could the Gs/GD virus lineage (H5N1) be the next pandemic strain?
- What is the mechanism(s) necessary for the current H5N1 virus to adapt further in humans?
- Could full switching of HA receptor specificity from avian- to human-type receptors or acquisition of binding affinity for long α2,6-sialylsaccharides evolve in bird species?
- How complex and diverse is the glycan topology in the respiratory tract of humans and pigs and in the gastrointestinal tract of birds?
- How long does it take for a potential pandemic influenza virus strain to acquire human transmissibility?
- Would the H5N1 virus retain its high pathogenicity in humans if it fully adapts to infect human upper-airway epithelia and would it produce a pandemic?
- Are there any distinct animal species in nature that play an intermediate role in the emergence of H5N1 variants as a source of new viruses able to produce future human pandemics?
- How widely is the current H5N1 virus distributed in nature, especially in animal species for which epidemiological surveys have not been done previously?
- What would be the most promising strategy for H5N1 control in the current situation where H5N1 is disseminated widely in both poultry and wild waterfowl?

Possible advances in the development of diagnostic testing and vaccines

In countries where the HPAI H5N1 virus is active, rapid diagnostic assays that distinguish influenza virus subtypes are needed to enable rapid initiation of appropriate therapy and infection-control measures, and timely epidemiological investigations [27,77]. Antigen-detection tests of clinical specimens are widely used for rapid diagnosis of seasonal human influenza, but are less useful for H5N1 influenza viruses because of their low sensitivity and the inability of

Table 1. Summary of the changing nature of the Gs/GD virus lineage (H5N1)

Category	Changing nature	Refs.
Gene	Emergence of multiple genotypes (combination of internal genes); A, B, C, D, E, P, V, W, X0-3, Y, Z and Z+[a]	[2,24,25]
Antigenicity	Emergence of multiple clades (HA gene); 0–10[b]	[4]
Ecology	Establishment of endemic infections in land-based poultry	[3,4]
	Persistence in a range of wild water and terrestrial birds	[4,30]
	Geographically large spread apparently by bird migration	[6,24,29]
Phenotype	Strong zoonotic properties	[27]
	Alteration of receptor-binding specificity	[25,32,66]
	Drug resistance (e.g. amantadine and oseltamivir)	[27]
Other	Acquisition of mammalian-type PB2-627Lys (commonly detected in a sublineage of genotype Z)	[29]

[a]Viruses with genotypes A, C, D and E have not been detected since 2002.

[b]Clades are further defined to the third order (e.g. clade 2.2.1).

validated tests to distinguish between influenza subtypes [27,77]. Several rapid diagnostic systems are being developed, including an H5-specific immunochromatography kit and high-speed real-time reverse transcriptase PCR (RT-PCR), with the latter as microfluidic continuous-flow RT-PCR [78]. However, the presence of multiple sublineages of current H5N1 viruses [22] and their changing nature pose challenges because assays need to be targeted at genes and epitopes that are continually evolving.

Furthermore, as the complex dynamics of influenza viruses in birds is constantly challenging species barriers, new technologies are required to determine whether a particular AI virus strain is adapting to human receptors [60]. Several new techniques have recently emerged for studying interactions of influenza viruses with host cell receptors. These include a dose-dependent direct binding assay [32,66] and a glycan microarray [60,61], allowing quantitative and high-throughput analysis, respectively. Although such technological developments still require improvements in simplicity and convenience for detection, future portable kits and instruments should allow field testing, adding a new dimension for characterizing and assessing AI outbreaks [60].

Mass vaccination of human populations with an appropriate vaccine is an effective way to reduce the spread, morbidity and mortality of pandemic viruses [2,27]. The new H5 sublineage in Egypt could be considered an important seed-virus candidate for such a vaccine [32]. However, the most important strategy is to minimize the potential for emergence of a pandemic strain and to limit the opportunity for humans, poultry and pigs to be infected by AI viruses. The main control for HPAI outbreaks is identification and depopulation of infected and exposed flocks. This 'stamping-out' approach is very effective in settings where the virus is still not widespread or is maintained in only confined poultry populations [4]. However, once a virus becomes endemic in wild bird populations, control and eradication become greatly complicated, especially in rural areas where complete confinement cannot be controlled and random rearing of backyard flocks is common [4]. Current genetic diversification of the Gs/GD sublineage has caused antigenic variation among different clades and even within the same clade. Thus, antigenically distinct viruses are now co-circulating in an endemic area, as observed in Egypt, where the crossreactivity of different sublineage viruses was too low for one of the viruses to be considered as a seed virus for vaccine antigen, even though the viruses were all isolated in the same geographic area [79].

Vaccination of poultry is now considered to be a preventive or adjunct control measure in several countries [27,80]. Proper vaccination can substantially help to control AI outbreaks only when administrated as part of an integrated control program that includes intensive surveillance, stamping out, quarantine, animal movement control and improved biosecurity [27,80]. New vaccines are being developed, including a reverse genetics vaccine, viral vectored vaccines, a virus-like particle vaccine, and a plasmid DNA vaccine [77,81]. The most promising method for responding rapidly to a possible outbreak and pandemic is the use of a plasmid-based reverse genetics system to construct a vaccine seed virus [81]. However, seed-virus selection must be revised periodically to produce well-matched and efficacious vaccines.

Concluding remarks

Although the current H1N1 pandemic [7,19] may have diverted attention from the continuing worldwide circulation of the H5N1 virus, the pandemic threat of H5N1 is still alarming. With the important role of wild birds in the epidemiology of current HPAI H5N1 viruses [3,4], continuing large-scale surveillance of AI viruses in wild birds, as well as in humans, poultry, and pigs, is crucial to an understanding of the evolution and global spread of these viruses [3,4,25,32]. Geographically widespread and complex dynamics of current H5N1 viruses also make control with a unified regime more difficult. Public health guidelines for control need to be tailored to meet local agricultural practices and people's awareness in the region.

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