Trends in Microbiology

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Review

Epidemiology, Evolution, and Pathogenesis of H7N9 Influenza Viruses in Five Epidemic Waves since 2013 in China

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H7N9 influenza viruses were first isolated in 2013 and continue to cause human infections. H7N9 infections represent an ongoing public health threat that has resulted in 1344 cases with 511 deaths as of April 9, 2017. This highlights the continued threat posed by the current poultry trade and live poultry market system in China. Until now, there have been five H7N9 influenza epidemic waves in China; however, the steep increase in the number of humans infected with H7N9 viruses observed in the fifth wave, beginning in October 2016, the spread into western provinces, and the emergence of highly pathogenic (HP) H7N9 influenza outbreaks in chickens and infection in humans have caused domestic and international concern. In this review, we summarize and compare the different waves of H7N9 regarding their epidemiology, pathogenesis, evolution, and characteristic features, and speculate on factors behind the recent increase in the number of human cases and sudden outbreaks in chickens. The continuous evolution of the virus poses a long-term threat to public health and the poultry industry, and thus it is imperative to strengthen prevention and control strategies.

Trends

The emergence of highly pathogenic H7N9 avian influenza virus is a potential threat to the poultry industry and to public health.

Elevated numbers of human cases of H7N9 virus infections during the fifth epidemic wave has meant the spread of H7N9 to several western provinces for the first time.

The evolutionary relationships of the HA and NA genes of H7N9 viruses during the five epidemic waves are examined here.

The dual receptor binding capacity of the H7 protein, along with the enhanced receptor affinity of the N9 protein, may contribute to the higher human infectivity of the H7N9 virus.

To reduce the risk of H7N9 human infection, we have to reduce or eliminate infections in poultry. Therefore, prevention and control strategies including strengthened biosecurity and improved outbreak management, as well as the prudent use of vaccines in poultry, should be reviewed and implemented.

A Brief Introduction to H7N9 and Other Recently Emerged Avian Influenza Viruses in China

Influenza A virus (IAV) is an enveloped, segmented, negative-strand RNA virus of the Orthomyxoviridae [1–3]. Wild waterfowl are reservoirs of IAVs; however, IAVs have a broad host range, including humans, wild birds, poultry, pigs, dogs, cats, horses, mink, pikas, and marine mammals [4–7]. The segmented nature of the IAV genome and the lack of proofreading activity of the viral polymerase allow for nucleotide changes to accumulate via antigenic shift and drift, resulting in added diversity and the emergence of novel influenza viruses [8]. The 1997 H5N1 and the 2013 H7N9 avian influenza virus (AIV) outbreaks in Hong Kong and mainland China are two familiar examples of the emergence of novel strains [9–16]. After H5N1 infection was reported in humans, the virus became enzootic in China, spreading to Europe and Africa via central Asia, resulting in 858 human infections and 453 deaths as of April 9, 2017[i]. In 2013, the novel H7N9 virus emerged in Eastern China, causing sporadic human infections, including fatalities. Since the first report of H7N9 infections in humans, there have been five epidemic

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waves[ii] with the fifth wave beginning in October 2016. Since then the number of reported cases has increased significantly, reaching a total of 466 in April 2017 [17,18]. The number of confirmed infections in the fifth wave exceeded that of previous waves, with a case fatality rate (CFR) of around 38% [data derived from the National Health and Family Planning Commission of PR China (NHFPC)[iii] and the World Health Organization (WHO)[iv]].

China offers an ideal environment for the emergence and circulation of novel influenza viruses with cross-species transmission potential [19]. This is because of its unique ecosystem, consisting of large quantities of domestic waterfowl, intermingled with various other animal species and poultry, as well as live-poultry markets (LPMs) [20]. There are at least six subtypes of AIV widely circulating in poultry in China, including H9N2 [21], H7N9, H5N1, H5N6, H6N1, and H10N8 [8,22–24], with the H5N1 and H7N9 AIV subtypes posing the highest threat to public health. Importantly, the recently emerged highly pathogenic (HP) H7N9 avian influenza virus caused outbreaks in chickens, leading to a large number of chicken deaths in several provinces in China[v] (see Figure S1 in the supplemental information online). Due to the rapidly increasing numbers of H7N9 human infections during the fifth epidemic wave, and the emergence of HP H7N9, the potential for a pandemic is of great concern [25]. Moreover, there have been various reassortment events involving the neuraminidase (NA) gene in the genetic backbone of the classical Asian HP H5N1, leading to novel H5NX variants such as H5N5, H5N6, H5N8, and H5N9, among others, that still pose a potential threat to the poultry industry and public health [26–29]. In addition, the novel H5N6 has replaced H5N1 as the dominant AIV subtype in southern China, and it has formed many distinct genotypes derived from continuous evolution and reassortment events. Importantly, 16 human H5N6 cases have been confirmed, as of April 2017, in mainland China[vi]. Therefore, an examination of the factors that resulted in the emergence and outbreak of this virus and other novel avian influenza viruses in China is urgently needed.

In a previous study, we proposed an ecosystem model for the emergence and cross-species transmission of novel AIV reassortants in China [8]. In China, and in many Asian countries, LPMs provide an important ecosystem for the circulation and evolution of AIVs [30,31]. During the long-distance transportation of live poultry to large wholesale markets or to distribution centres different avian influenza viruses mixed and shared their genetic materials, and thus generate novel variants. In addition, asymptomatic live poultry, transported across the country, allows for the rapid dissemination of novel viruses on a nationwide scale, resulting in extensive human exposure [19]. In southern China, the main driving force for animal sales in LPMs is human consumption, which allows a high frequency of human–poultry contact [32–34]. Of note, most human H7N9 cases have been associated with previous exposure to poultry and/or to LPMs. Moreover, H7N9 viruses have been extensively detected among poultry and in the environment in LPMs across China [32,35] {average isolation rate of 3.0% from 15 cities across 5 provinces between 2013 and 2014 [36] and from different poultry (e.g., pigeons, chickens, geese, and ducks) sold at LPMs}. Thus, poultry is considered to be the major source of H7N9 infections in humans [30,37–39].

In addition, live poultry species tend to be housed adjacent to numerous other animal species, including pigs, dogs, cats, ferrets etc. in southern China. These species are susceptible to influenza viruses and can serve as 'mixing vessels' for reassortment [40], leading to the emergence of novel infectious variants that are pathogenic to humans [8,40,41]. Infections of humans may result in host adaptation of H7N9 and/or reassortment with endemic human/swine influenza viruses. Such a scenario would be an enormous threat to public and global health as mammalian receptor-adapted H7N9 viruses could spread among humans. Therefore, systematic surveillance of AIVs in LPMs in China is essential for the detection of novel reassortant viruses and the potential for interspecies transmission. Our review aims to compare

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the different epidemic waves of H7N9 influenza virus with regard to its epidemiology, pathogenesis, evolution, and characteristic features, which may contribute to the higher number of human cases and the sudden outbreaks in chickens in the fifth wave.

Epidemiological Characteristics of Human Infections with H7N9 Influenza Virus

The 2013 H7N9 AIV likely emerged in Zhejiang or Jiangsu in the Yangtze River Delta, although it was first recognized in Shanghai [13,19,42]. Until April 9, a total of 1344 human infections with H7N9 influenza virus had been reported in China, with 511 (data from Centers for Disease Control of China) being fatal. The epidemic waves span from October 1 until September 30 of the following year, as defined by the WHO[ii]. H7N9 infection cases decreased in wave 4 (from October 1, 2015 to September 30, 2016) (Figure 1A). However, a higher number of cases were detected in wave 5 compared to any of the previous waves. Notably, there was an approximately sevenfold increase in cases in Jiangsu province in wave 5 compared to wave 4 [18]. H7N9 was first detected in the Yangtze River Delta Region, which includes Zhejiang, Jiangsu, and Shanghai [13,42–45]. This region had the highest number of H7N9 cases. The second largest number of cases was detected in Guangdong, the Pearl River Delta Region, with 256 H7N9 human infections (Figure 1A). Interestingly, a regional expansion of H7N9 influenza infection events was observed from eastern to western China (Figure 1A). In wave 5, H7N9 viruses were first detected in some western provinces (Chongqing city, Gansu, Sichuan, and Tibet). This indicates that, with the geographic shift of the epidemic, the prevention and control of H7N9 avian influenza virus in the future should not only include southern China but also include the western provinces[vii]. Male H7N9 infections (69%) tend to be higher than female infections [43,46] (Figure 1B). In terms of age, most of the cases include individuals between 40 and 60 years of age or more than 60 years of age [42,47,48]. A recent study showed that a change occurred in the distribution of cases during the fifth wave from the elderly to middle-aged adults, and from urban locations to semiurban and rural areas [49].

There are also other important epidemiological scenarios worth mentioning. In April 2013, a study first reported cases of coinfections of H7N9 and seasonal H3N2 IAV [50] (Figure 2A). This

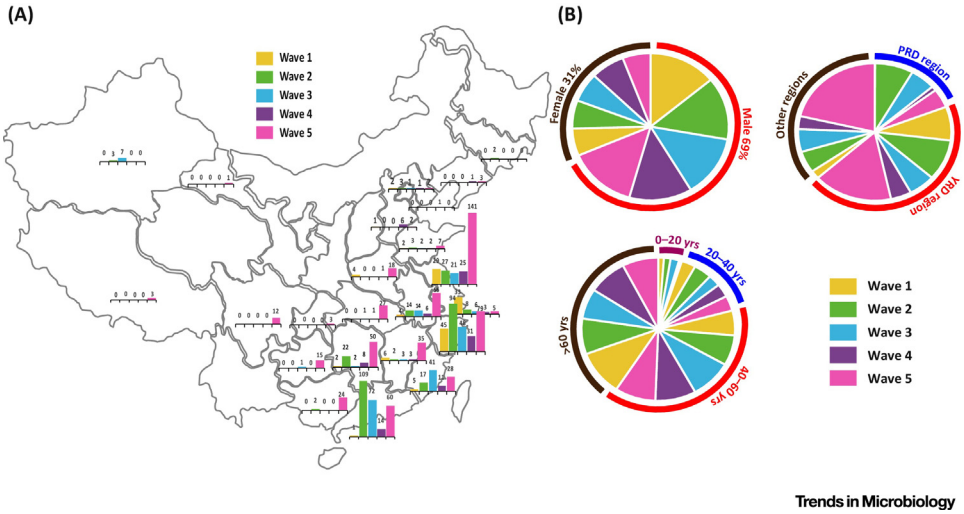
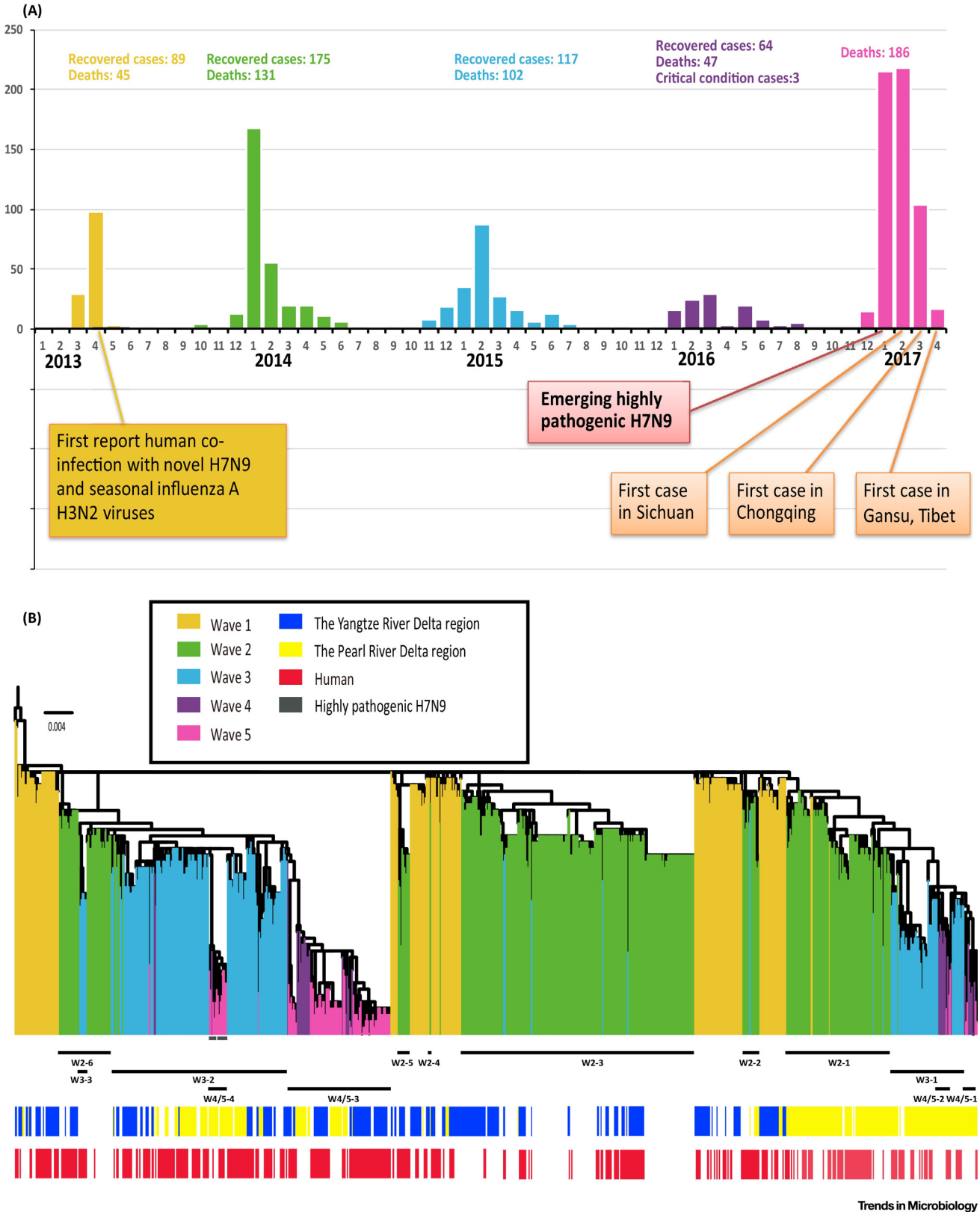


Figure 1. Distribution and Characteristics of Human H7N9 cases in Mainland China. (A) Geographic distribution of human H7N9 cases in mainland China from February 2013 to April 2017. (B) Comparison of human H7N9 case distribution in terms of gender, region, and age in the five epidemic waves. YRD Region: Yangtze River Delta Region; PRD Region: Pearl River Delta Region. Reference H7N9 infection cases from each wave are denoted by different colors. (Data from Centers for Disease Control of China.)



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shows that humans might act as mixing vessels for virus reassortment, which might facilitate human-to-human transmission [50]. H7N9 epidemics in mainland China pose a threat to residents of other countries. Two individuals travelling in China, with a history of being in contact with live poultry and copious droppings, were confirmed H7N9-positive after returning to Canada in January 2015 [51]. In addition, two human cases were reported with infections with a novel HP H7N9 avian influenza virus at the beginning of wave 5 (Figure 2A) [25]. HP H7N9 viruses, which were first reported 4 years after the emergence of low-pathogenic (LP) H7N9 virus in China, harbour multiple amino acid insertions in the HA protein cleavage site [25] (Figure 2A). Overall, in the fifth wave, the diffused distribution area of the H7N9 influenza virus epidemic, the significantly increased number of human cases, and the recent HP H7N9-infected cases have attracted the public's attention. An important question to answer is whether HP H7N9 viruses pose an increased threat to public health in the future.

Phylogeny, Evolution, and Mutation of H7N9 Influenza Virus in China

An important question to answer is whether HP H7N9 viruses pose an increased threat to public health in the future. To address this point, we first analysed the evolutionary relationships between the hemagglutinin (HA) and NA genes of H7N9 isolated from waves 1–5, including the HP H7N9, and summarized the mutations associated with host adaptation or resistance.

Evolutionary Relationships of the Hemagglutinin Genes of H7N9 Viruses during Epidemic Waves 1–5

Early H7N9 viruses from several provinces have similar surface genes [15,38,52–54] and they formed a highly polytomous clade, which suggested the dispersal of near-identical viruses from a common source [15,38]. Moreover, the viruses in wave 1 are most closely related to the first-discovered virus of the lineage (A/Shanghai/1/2013), in agreement with previous studies (Figures 2 B and 3 A) [19,55].

Epidemic waves 2 and 3 started in the early autumn of 2013 and 2014, respectively, both lasting 9 months (Figure 2A). Waves 2 and 3 H7N9 viruses derived from the original major genotype of wave 1 and evolved into different lineages that disseminated to a wider region of China than did viruses of wave 1 [38,56–59]. Viruses of wave 3 derived from wave 2 viruses and thus clustered together [38]. Wave 2 viruses can be divided into six clades: W2-1, W2-2, W2-3, W2-4, W2-5, and W2-6 (Figure 2B). Given that viruses of the six clades were all detected in wave 1, we can conclude that they exclusively derived from this wave. Viruses in clade W2-1 were mainly isolated in the Pearl River Delta Region (Figure 2B), while viruses of other clades of wave 2 (W2-2, W2-3, W2-4, W2-5, and W2-6) were isolated in multiple provinces (Figure 2B). The majority of viruses from wave 3 clustered into three clades (W3-1, W3-2, W3-3) and circulated in different regions. Viruses in clade W3-1 mainly circulated in the Pearl River Delta Region and derive from clade W2-1 (Figure 2B). All of these results are in agreement with a previous study [55].

The current fifth epidemic wave began in October, 2016, and had the highest number of deaths (186 deaths as of April 9, 2017) (Figure 2A). Wave 4 and 5 viruses clustered together, forming

Figure 2. Epidemiology and Evolution of the HA Gene of the H7N9 Virus in the Five Epidemic Waves. (A) The number of H7N9 human cases in the five epidemic waves and the number of recovered cases and deaths until April 9, 2017 (data from the Centers for Disease Control of China) are shown for waves 1–4 in yellow, green, light blue, purple, and pink, respectively. Highly pathogenic H7N9 emerged at the end of December, 2016, with the first reports of human cases occurring in Sichuan, Chongqing, Gansu, and Tibet. (B) Maximum likelihood (ML) phylogeny of the H7N9 influenza virus HA gene. The HA gene sequences from all five H7N9 waves were downloaded from Global Initiative on Sharing All Influenza Data and GenBank for phylogenetic analysis. ML trees were inferred with the software RAxML under the GAMMA GTR model with 1000 bootstraps and A/Shanghai/1/2013 as the root. Reference H7N9 viruses from each wave are denoted by different colors. HP avian influenza viruses (AIVs) are shown in dark purple. Colored boxes below the tree indicate the regions and species from which the corresponding H7N9 viruses derive. H7N9 viruses isolated from the Yangtze River Delta Region, the Pearl River Delta Region, and other regions are shown in blue, yellow, and no-color, respectively. The red/no-color denote the human/nonhuman H7N9 virus.

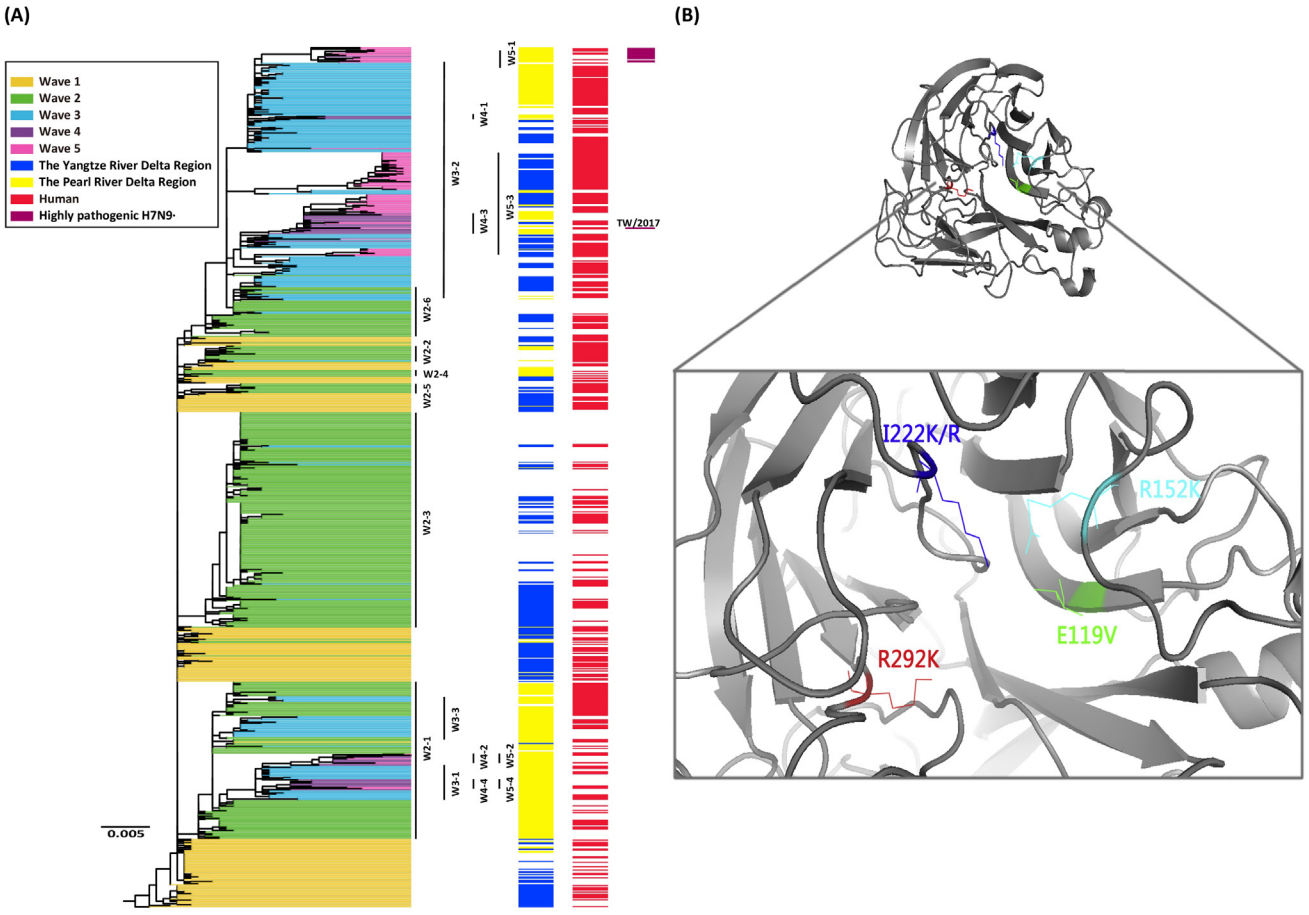

Figure 3. H7N9 Virus Evolution of the NA Gene in the Five Epidemic Waves and Drug-Resistant Sites of the H7N9 NA Protein. (A) Phylogenetic tree of the H7N9 influenza virus NA gene. The NA gene sequences were downloaded from Global Initiative on Sharing All Influenza Data and GenBank. ML trees were inferred using RAxML under the GAMMA GTR model with 1000 bootstraps. Reference H7N9 viruses from each wave are denoted by different colors. HP avian influenza viruses (AIVs) are shown in dark purple. Colors on the right of the tree indicate the regions and species from which the corresponding H7N9 viruses derive. H7N9 viruses isolated from the Yangtze River Delta Region, the Pearl River Delta Region, and other regions are shown in blue, orange, and no-color, respectively, and the red/no-color denotes the human/nonhuman H7N9 virus. (B) Drug-resistant mutations in NA of the human H7N9 virus. The 3D structure template was downloaded from SWISS-MODEL[xi]. Images were created with PyMOL (version 1.5.0.4).

four different clades (W4/5-1, W4/5-2, W4/5-3, and W4/5-4) based on analysis of the HA gene. Viruses of wave 4 were derived from wave 3 viruses and were mainly isolated from the Pearl River Delta Region with rare isolations in the Yangtze River Delta Region or other areas (Figure 2B). Viruses in wave 5 emerged from wave 4 viruses and were isolated in both the Pearl River Delta and Yangtze River Delta Regions. Of note, novel HP H7N9 viruses that contained the basic KRKRTAR/G or KGKRIAR/G motifs emerged at the end of December, 2016, in Guangdong [25]. All of the HP H7N9 sequences available so far from Global Initiative on Sharing All Influenza Data (GISAID) and GenBank were isolated from the Pearl River Delta Region, indicating that this area is the most likely source of this virus. These HP H7N9 viruses derived from viruses of wave 4 that circulated in the Pearl River Delta Region and formed an independent wave 5 cluster (W5-4) based on the phylogeny of the HA gene (Figure 2B). Of note, some HP H7N9 viruses have also been isolated from chickens, which are likely the source of the human infections. Moreover, both human- and avian-isolated HP viruses clustered together. Given the increasing numbers of H7N9 outbreaks in chickens, and the current ongoing fifth

epidemic in mainland China, future monitoring of animals and gene analysis of HA and other genes of both HP and LP H7N9 viruses need to be strengthened.

Evolutionary Relationships of the NA Gene and the Six Internal Genes of H7N9 Viruses during Epidemic Waves 1–5

The phylogeny of the NA gene of H7N9 viruses of all five waves exhibited a similar topology to that of the HA gene (Figure 3A). However, the HP H7N9 viruses formed an independent cluster based on phylogeny of the HA gene, while one HP H7N9 strain (A/Taiwan/1/2017) was separated from the others based on NA phylogeny. This suggests that H7N9 virus (including HP H7N9) underwent continuous evolution through reassortment. Interestingly, some viruses in wave 5 derived from wave 3 based on the NA phylogeny, which is different to the HA gene. In addition, viruses from waves 4 and 5 formed several different groups and displayed more complex relationships compared to the HA gene.

Several studies concluded that the H7N9 virus derived by reassortment between H7 and N9 viruses (surface genes) of wild birds and poultry H9N2 virus (internal genes) [14,52,60]. After the H7 and N9 virus introduction to chickens, reassortment took place with enzootic H9N2 viruses, adopting their internal gene complex to form the H7N9 virus that emerged in 2013 [14,15,52–54,60]. Previous studies analyzed the internal genes of H7N9 viruses and revealed that the internal genes formed multiple genotypes and derived from different H9N2 sub-lineages [15,38,52–54]. Another study analyzed the evolutionary relationships of six internal genes of the previous three epidemic waves of H7N9 viruses and showed that the PB2 and PB1 genes were divided into four major clades; the NP and M genes were divided into three clades; and PA and NS clustered into two major clades [55]. The genotypic diversity may be caused by sequential reassortment events after the viruses were introduced to different regions, or by several distinct reassortment events occurring approximately simultaneously when the viruses were initially generated [19]. Of note, a recent study reported that H7N9 virus is a reassortant between H5N6 and H6N6, which further increases the genetic diversity of the H7N9 virus [61].

Host Adaptation and Resistance Mutations during Epidemic Waves 1–5

In order to understand H7N9 influenza virus evolution, selected amino acid mutations on HA and PB2 associated with mammalian adaptation were analyzed. In the five epidemic waves, most viruses contained the G186V and Q226L/I substitutions in the HA gene, which are the major contributors to the high-affinity binding of this virus to human receptors [55,62,63]. In addition, a hemagglutinin G225D substitution within the receptor binding site, which binds preferentially to α2,6-linked sialic acids (SAs), and may increase the likelihood of upper respiratory tract transmission [64], was reported in the A/Fujian/18/2015 viral strain isolated from family clusters of H7N9 influenza infections [65]. Five out of 551 available HA sequences from GISAID include this substitution. Notably, a signature of highly pathogenic avian influenza viruses (the insertion of basic amino acid residues RKRT at the cleavage site connecting the HA1 and HA2 peptide region) was found in six viruses isolated from human cases in wave 5 in Guangdong Province[viii]. These mutations may also increase the virulence of influenza virus in poultry [66] and cause more and more outbreaks and a larger number of deaths in chickens in 2017[ix]. In addition, the majority of human-origin H7N9 viruses of each wave acquired a PB2-E627K mutation, which increases pathogenicity in mice [17]. Compared to previous waves, the proportion of a A588V substitution in the PB2 protein, which confers enhanced pathogenicity in mice [67], increased to 77% (94/122) in the fifth epidemic wave (as of April 2017)[viii].

In H7N9 AIV patients treated with NA inhibitors (NAIs), resistant mutations have been detected, such as an R292K substitution in NA that confers resistance to oseltamivir [68–73]. Based on

the available gene sequences of human H7N9 isolates, we found that 24 out of 606 viruses contain the R292K substitution (including some recently emerging HP H7N9 virus) [25]. However, 292K was not found in avian-origin H7N9 viruses. Additional NAI-resistant substitutions, such as NA E119V (4/592), R152K (4/593), and I222K/R (1/597), were also previously reported in the H7N9 subtype [74,75]. E119 and I222 are critical residues supporting the framework of the NA-binding pocket, while R292 and R152 participate in catalysis [76] (Figure 3B). The R152K mutation had been shown to confer mild resistance to zanamivir and oseltamivir *in vitro*. The E119V and NA I222K/R mutations confer different levels of resistance to peramivir, zanamivir, and oseltamivir, and NAI treatment does not inhibit H7N9 replication if the virus possesses these substitutions [74]. Fortunately, the E119V and NA I222K/R mutations have not yet been found in poultry, and thus the circulating H7N9 should still be sensitive to NAI drugs.

Virology

In the following sections we describe the pathogenicity of both HP and LP H7N9 in humans and animals, as well as the receptor-binding characteristics of these viruses.

Pathogenicity

Humans. Up to April 9, 2017, 38% (511/1344) of humans with influenza A (H7N9) infections within the five epidemics died (Table 1 and Figure 2A). Most patients developed fever, cough, and weakness [43,77]. Three patients (two from Shanghai City and one from Anhui province) were the first confirmed cases carrying a novel avian-origin influenza A (H7N9) virus infection in 2013 [13], with a long time interval between the onset of illness and laboratory confirmation. During the previous four waves, 85% (659/775) of patients had a history of exposure to live poultry [77], and some patients experienced complications, including pneumonia, respiratory failure, or acute respiratory distress syndrome [42,43,77]. The time between the onset of illness and admission to hospital was 5 [Inter-Quartile Range (IQR): 4–7] days, 5 (IQR: 3–7) days, and 5 (IQR: 3–7) days for waves 1–3, respectively. The time between the onset of illness and laboratory confirmation was 8 (IQR: 6–11), 8 (IQR: 6–11), and 8 (IQR: 6–10) days for waves 1–3, respectively [43,77]. In wave 4, the time from illness to death was shortened by 6 days compared to wave 1. Moreover, there were 775 cases and 325 deaths (case fatality rate: 42%) in mainland China.

As of April 9, 2017, 571 cases have been reported in the fifth wave, with a CFR of ~40% (186/571). In general, the time from the onset of illness to death was 14 days (8–21) (Table 1), and most patients had a history of contact with live poultry in the Jiangsu province [18]. During the fifth wave, the emergence of an HP H7N9 influenza infection in humans was first reported in Guangdong Province [25]. Notably, disease progression in these two patients was quick, but resolved rapidly after treatment with influenza-specific antivirals [25]. In addition, a recent report showed that HP H7N9 patients were hospitalized earlier (6 days) than LP H7N9 patients (13–14 days) and were more likely to have had exposure to sick and dead poultry in rural areas [78]. This study also suggested that, given the higher mortality, and the shortened intervals between onset of illness and diagnosis and death, there was a more rapid progression and greater disease severity for HPAI A (H7N9) case-patients compared to LPAI A(H7N9) case-patients; however, these differences were not statistically significant due to the small number of cases [78]. The pathogenesis of HP H7N9 in humans requires further investigation. Of note is that most human cases had underlying medical conditions, which makes understanding the pathogenicity of these viruses difficult [42,43,77]. In addition, epidemiologic surveys suggest that there have been subclinical/asymptomatic or mild human infections with H7N9 viruses [79–81], and that the enhanced virulence of HP H7N9 may increase the morbidity for humans [82]. So, whether or not the HP H7N9 virus leads to a sudden increase [18,46] in cases requires further investigation.

Table 1. Comparison of Live Poultry Exposure, Clinical Signs, Case Fatality Rates, Median Time of Hospitalization, and Median Time to Death of H7N9 Cases

		Live poultry exposure	Main clinical symptom	Underlying condition	Case fatality rate	Illness onset to hospitalization (median time)	Illness onset to laboratory confirmation (median time)	Illness onset to death (median time)	Refs
Waves 1–4	Wave 1	85% (659/775)	Fever, cough, weakness, muscle soreness, shortness of breath, chest distress, nausea, etc.	53% (289/545)	34% (45/134)	5 (4–7)	8 (6–11)	21 (11–34)	[13,43,77]
	Wave 2				42% (131/306)	5 (3–7)	8 (6–11)	19 (10–31)	
	Wave 3				45% (102/219)	5 (3–7)	8 (6–10)	16 (10–27)	
	Wave 4				41% (47/114)	4 (3–6)	8 (6–11)	15 (8–24)	
Wave 5	Wave 5-general	90% (442/500)[a]	Fever, cough, chills, pneumonia	54% (234/432)	33% (186/571)[b]	3 (2–5)	8 (6–10)	14 (8–21)	[18,25,46,78]
	HP (highly pathogenic)	100% (8/8)		63% (5/8)	50% (4/8)	2 (0–5)	6 (4–9)	6 (5–44)	

[a]The number of LP H7N9 cases in wave 5.
[b]The number of wave 5 infected cases, both low-pathogenic (LP) and HP, until April 9, 2017.

Box 1. Pathogenicity and Transmission of H7N9 in Animals

The pathogenicity of LPAI H7N9 virus was experimentally analyzed in chickens and ducks. Although chickens were easily infected with H7N9, and could transmit the virus to naïve animals by direct contact, the clinical presentation was consistent with low-pathogenic avian influenza virus (LPAIV) infection regardless of breed or dose [104,105]. No mortality or morbidity was observed in infected chickens [106–108]. Chickens in direct contact with H7N9-infected birds shed virus from the cloaca from day 5 postinfection (p.i.) to day 8 p.i. [107]. Ducks could be infected by high-dose challenge, shedding an average of 4.8 $\log_{10}$ EID_{50}/ml on days 2 and 4 p.i., but showed no clinical signs, and shed relatively low numbers of viruses for shorter periods of time than did chickens [108].

Interestingly, H7N9 was highly virulent in mice, leading to severe (≥20%) body weight loss and mortality [84,109,110]. Belser *et al.* [109] found high viral titers on days 3 and 6 p.i. in lungs (≥$\log_{10}$ 5.5 PFU/ml) and nose (≥$\log_{10}$ 2.2 PFU/ml), but no virus was detected in the brain on day 6 p.i.

Pigs were productively infected by H7N9, shedding virus as early as 1 day p.i. and lasting for 5–6 days. Peak virus titers ranged from 3.49 to 5.16 $\log_{10}$ $TCID_{50}$/ml [111]. However, there was no transmission to the direct-contact pigs or airborne-exposed pigs and ferrets [45,111,112].

In guinea pigs, H7N9 displayed a number of mammalian adaptive traits, including efficient replication and transmission. Virus was detected in nasal washing fluid, grew in the upper respiratory tract at high titers, and transmitted to all direct-contact guinea pigs [113].

H7N9 was efficiently transmitted between ferrets via direct contact, but less efficiently by airborne exposure. H7N9 also replicated efficiently in the ferret respiratory tract. Belser *et al.* [109] found mean peak titers ranging from $\log_{10}$ 5.4 to 6.2 PFU/ml in nasal turbinates and from $\log_{10}$ 5.0 to 6.5 PFU/g in lungs. However, ferrets exhibited relatively mild clinical signs compared to infection with highly-pathogenic avian influenza viruses such as H5N1, and infection with H7N9 was rarely fatal [45,83,84,109,111,114,115].

Animals. Given that most of the severe and fatal H7N9 cases were in older individuals with pre-existing medical conditions, and that surveillance did not focus on asymptomatic or mild-symptomatic H7N9 cases, it is difficult to establish the real pathogenicity of the H7N9 strain in humans based just on epidemiological data. Various animal studies have been performed to address this in chickens, ducks, mice, pigs, guinea pigs, and ferrets (Box 1).

There are currently no data showing the impact of HP H7N9 viruses harboring HA mutations in the cleavage site on pathogenicity in animal models, although they have caused a large number of deaths in chickens within a short period of time. More studies are required to determine the species involved in the natural transmission of these HP viruses for which epidemiological surveillance has to be strengthened, and more animals need to be screened as part of monitoring programs (see Outstanding Questions).

Receptor Binding Characteristics

In agreement with the findings in human and animal studies, *in vitro* and *ex vivo* studies showed that H7N9 HA exhibits dual-receptor specificity and has affinity for both human-type, α2, 6-linked SA and avian-type, α2, 3-linked SA receptors [62,83,84] (influenza A virus receptor binding: see Box 2). Recent research shows that HP H7N9 had a slightly increased binding capacity for both receptors compared with LP H7N9 [82].

This dual specificity is probably beneficial for continued virus dissemination among chicken and human populations and the appearance of more pathogenic variants, like the recently emerged HPAI H7N9 [17,25,66]. However, a previous study showed that the prototypical H7N9 virus that results in severe acute lower-respiratory-tract infection has limited binding to human receptors in the upper airway, in contrast to other human-adapted viruses like the pandemic H3N2 [85]. Therefore, human infections with H7N9 remain sporadic since the viruses have not yet gained efficient transmissibility among humans to raise a pandemic alert.

The HA Q226L and G186V (H3 numbering throughout) mutations increase the affinity of H7N9 viruses for α2,6 receptors [63,85–87]. Structurally, these key site variations probably improve

Box 2. Functions and Distribution of Sialic Acid Receptors on Virus Host Species Selection (Specification)

The ability of influenza A viruses to infect a specific host is mainly determined by the recognition and binding of the HA proteins to specific sialic acid (SA) receptors on the cell surface [116]. Therefore, different SA receptor patterns are one of the determinants of viral tissue tropism and host range. SA refers to neuraminic acid derivatives of negatively charged 9-carbon monosaccharides typically located at the terminal moieties of N- or O-linked glycoconjugates. The two predominant members are *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), which differ only at the C5 position [117]. The spatial conformation of the SA given by the glycosidic bond between the C2 site and the penultimate galactose (α2,3 and α2,6 linkages) specifies the viral receptor binding propensity (Figure I). For example, avian influenza A viruses preferentially connect to SA in the α2,3 linkage (SA2,3Gal), which is mainly distributed in the intestinal tract of birds, while human-adapted influenza A viruses selectively bind to SA in the α2,6 lineage (SA2,6Gal), which is mainly prevalent in the human upper respiratory epithelia. Experimentally, characterization of the viral receptor binding property involves methods such as hemagglutination-based assays through specific sialidase treatment on horse, guinea pig, chicken, turkey, or goose red blood cells with differential expression of SA2,3Gal and SA2,6Gal, and solid-phase enzyme-linked receptor-binding assays by using synthetic sialylglycopolymers with distinct α2,3/α2,6 linkage between the SA termini and the second last saccharide [118]. Alternatively, tissue sections, such as those prepared from the apical surface of the human trachea and the deep pulmonary alveoli, which primarily express physiologically relevant glycans of SA2,3Gal and SA2,6Gal, respectively, could also be stained for distinct influenza viruses to analyze the receptor binding avidity [85].

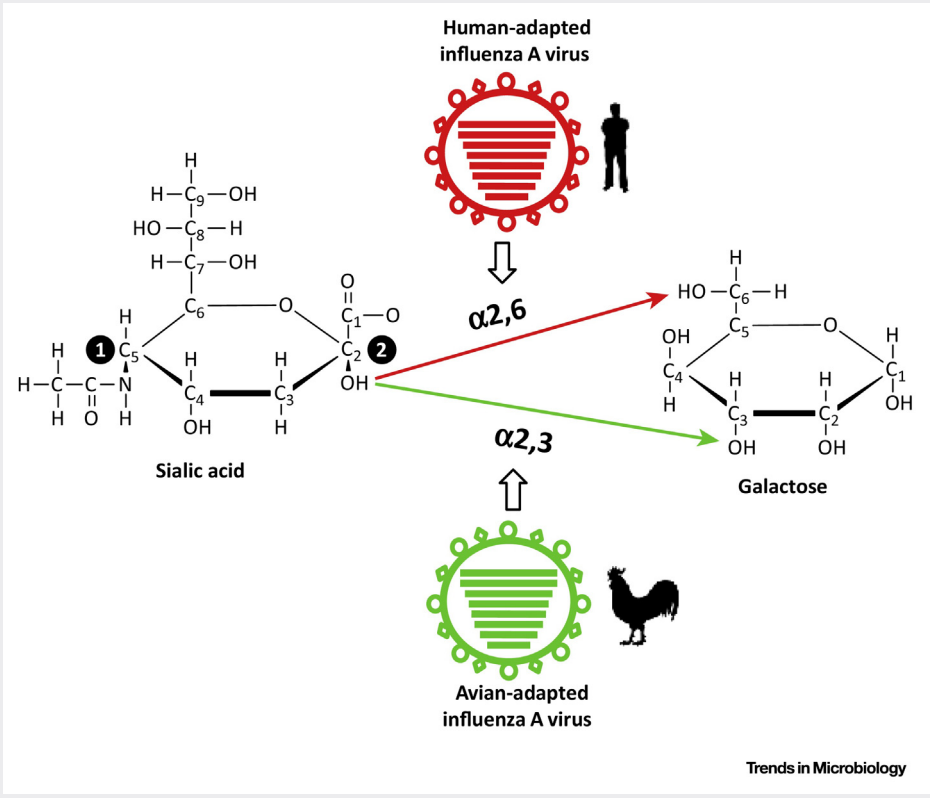

Figure I. Structure of Sialic Acid and Glycosidic Bonds to Neighbouring Galactose. ① corresponds to the *C*5 position of *N*-linked acylation (H3COCHN-) or glycolylation (HOH2COCHN-), and ② corresponds to the *C*2 position of the α2,3- or α2,6-linkage between sialic acid and contiguous galactose.

the conformational constraints in the receptor binding site to provide a more flexible interaction with human-type receptors [85]. Apart from HA, the NA protein also plays a role in H7N9 virus receptor binding. In particular, N9 still possesses a secondary haemadsorption (Hb) site for SA binding other than the traditional sialidase site for enzymatic catalysis. Additionally, N2 with a D151G substitution and N1 with a G147R substitution have also been shown to possess receptor binding properties [88–90]. Recent studies showed that purified N9 proteins and

reassortants, on the basis of zoonotic H7N9, binds to turkey red blood cells and synthetic receptor mimics to the Hb site and, marginally, to the sialidase site. Moreover, the Hb site mediates avidity to SA 2,3Gal and SA 2,6Gal analogues without preference for receptor species, while the catalytic site specifically enables enhanced binding to just human-type SA receptors [91].

Unlike the Asian HP H5N1 that caused 858 laboratory-confirmed human cases over two decades of circulation, the novel H7N9 virus has already caused a total of 1344 human infections in just a 4-year period, with a sharp increase in the fifth epidemic wave until April 9, 2017. Although the molecular mechanism that enables zoonotic influenza viruses to invade, and then transmit among humans, remains incompletely understood, acquiring the binding ability to human-like receptors is a prerequisite. Whether the abovementioned dual-receptor binding capacity of the H7 protein containing the crucial amino acid marker 226L, plus an evident enhancement of receptor affinity owing to the N9 protein, jointly contribute to the higher human exposure rate of H7N9, in contrast to H5N1, requires further investigation.

Concluding Remarks and Future Perspectives

The H7N9 virus originated in poultry via reassortment and was introduced into the human population through LPMs [92–94]. Notably, along with the emergence of HP H7N9 influenza virus, the number of reported cases in the fifth epidemic wave was higher than in previous waves, suggesting that the H7N9 virus is still circulating widely in China. Moreover, during the fifth epidemic wave, the H7N9 virus is circulating in a larger geographic area in China (Figures 1 and S1), with cases of HP H7N9 infection associated with contact with sick and dead poultry in rural areas. Therefore, continuous surveillance of poultry, the environment, and humans for the presence of H7N9 viruses in both urban and rural China is imperative. In addition, mammalian host adaptation of the H7N9 virus, and reassortment generated by the cocirculation of other subtype influenza A viruses [both AIV and swine influenza virus (SIV)] in LPMs in the south of China, should be looked at more carefully.

Vaccination is one of the best approaches to control a variety of virus infections; however, no commercial H7N9 vaccine is available for poultry or humans. An H7N9 monovalent vaccine with the MF59-adjuvant has been reported to be tolerable and immunogenic in adults, and it induced potentially protective immune responses [95]. In addition, a recombinant H7N9-53TM virus was recently rescued that could be a better vaccine candidate against an H7N9 outbreak [96]. A new lipid nanoparticle (LNP)-formulated modified mRNA vaccine, encoding the HA proteins of H7N9 and H10N8 viruses, was reported recently to induce protective immunogenicity with acceptable tolerance profiles [97]. The timely improved vaccination programs aimed at preventing the H7N9 virus from evolving and causing a potential devastating pandemic should be eagerly encouraged. Notably, with the emergence of HP H7N9 virus which had low cross-protection against LP H7N9, the WHO recommended an HP H7N9 (A/Guangdong/17SF003/2016 (SF003)) as an additional human candidate vaccine virus [82]. To further minimize the risk of human infection with avian influenza A (H7N9) virus via exposure from poultry carriers, traditional whole-virus inactivated vaccines and genetically engineered live vector vaccines have been actively prepared against H7N9 in poultry, although the predicted immunogenicity of avian-origin H7 antigens seems comparatively limited [98]. It is noteworthy that, considering the current cocirculation of HP and LP H7N9 avian influenza viruses plus the frequent live poultry trade, especially in the Guangdong and Guangxi provinces, a recombinant bivalent inactivated avian influenza vaccine – comprising the Re-8 strain of H5N1 subtype and the H7-Re1 strain of H7N9 subtype, generated by reverse genetics on the internal gene backbone of the egg-adapted high-yield A/Puerto Rico/8/1934(H1N1) virus – has been initially authorized by China's Ministry of [x]. Moreover, drug-resistance mutations in H7N9 isolates

Outstanding Questions

Faced with the emergence of highly pathogenic H7N9 influenza virus, what kind of measures should the government take to control outbreaks and the spread of H7N9 influenza virus in China?

Will continuous cocirculation of H7N9 influenza virus and other influenza A virus subtypes generate novel reassortants with enhanced pathogenicity and transmissibility?

Will H7N9 viruses from the fifth epidemic wave with mutations in HA and PB2 exhibit higher pathogenicity in mammalian and avian hosts?

Is the different receptor binding capacity of H7N9, due to crucial mutations in HA and NA, responsible for the higher exposure rate of H7N9 compared to H5N1 in humans?

Is an H7N9 influenza vaccine in poultry urgently needed in China?

against NAI have already been reported [25,72,99] – which highlights the need for new classes of anti-influenza drugs [100–103].

To control H7N9 and other emerging AIVs, it is necessary to reconsider management of the agricultural and trading practices currently in place in China (see Outstanding Questions). Some measures for households, large-scale farms and LPMs – such as surveillance in poultry and the environment (use of sentinels), better management (e.g., preventing mixing of animals, setting up regular disinfection and hygiene checks), and preventing the geographical spread of H7N9 across provinces via quarantine of poultry – should be established across China. Furthermore, a vaccination approach is critical to control this virus in poultry. The long-term complete shutdown of LPMs should be considered if the HP viruses continue to emerge in poultry. The continuous evolution of the virus poses a long-term threat of H7N9 infection in humans in China, and thus strengthening prevention and control of H7N9 is imperative.

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Resources

[i] www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/
[ii] www.who.int/influenza/human_animal_interface/avian_influenza/riskassessment_AH7N9_201702/en/
[iii] www.nhfpc.gov.cn/
[iv] www.who.int/influenza/en/
[v] www.flu.org.cn/en/news_detail?action=ql&uid=&pd=&newsId=19378
[vi] www.chp.gov.hk/files/pdf/2017_avian_influenza_report_vol13_wk17_chi.pdf
[vii] www.flu.org.cn/scn/news-19069.html
[viii] http://search.agri.gov.cn/agrisearch/search.jsp
[ix] www.moa.gov.cn/zwllm/yjgl/yjcl/201705/t20170505_5596929.htm
[x] www.sohu.com/a/147950745_358939
[xi] https://swissmodel.expasy.org/

Supplemental Information

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