PUBLIC HEALTH RISK UPON HUMAN EXPOSURE TO HIGHLY PATHOGENIC AVIAN INFLUENZA H5N8

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ABSTRACT

Influenza A viruses are divided into subtypes based on two proteins on the surface of the virus: the hemagglutinin (HA) and the neuraminidase (NA). Influenza A viruses mainly affect avian species, but some highly pathogenic avian Influenza (HPAI) A subtypes can undergo reassortment or can acquire mutations, which can result in a novel virus strain capable of mammalian or even human infection. A recent outbreak of HPAI H5N8 in Korea has been reported with no human cases to date. However, other HPAI H5 viruses have been associated with human infection. Therefore, the potential public health risk upon human exposure to HPAI H5N8 was assessed in this review. The novel reassortant HPAI H5N8 is subdivided into group A and group B viruses based on genetic differences. Group A viruses were predominant during the initial outbreak, but group B viruses were predominant during a new outbreak in 2016, suggesting these viruses are the main threat to human health to date. The introduction of H5N8 viruses into Korea and the spread of H5N8 viruses to Europe, Russia and North America has been attributed to migration of wild birds, suggesting rapid geographical spread of HPAI H5N8 viruses and limited surveillance. H5N8 viruses were able to affect both avian species and mammalian species and pathogenicity was considered mild in both avian and mammalian species. However, while horizontal transmission in avian species was efficient, horizontal transmission in mammalian species was unsuccessful. HPAI H5N8 viruses preferentially bound avian-like receptors and showed insufficient replication in human cells, indicating that HPAI H5N8 are not fully adapted to mammals. Findings suggested a difference between group A and group B viruses, with group B viruses being more pathogenetic and more adapted to humans than group A viruses. Additionally, currently circulating HPAI H5N8 viruses were shown to be susceptible to NAIs and certain vaccines were shown to protect against H5N8 infection. However, susceptibility to treatment can be altered when mutations occur. These results together suggest that the public health risk of the HPAI H5N8 strains is low. However, the rapid geographical spread of HPAI H5N8 viruses, their ability to infect various avian and mammalian species without causing clinical signs and the tendency of influenza A viruses to mutate and reassort are major concerns. Therefore, it seems of interest to extensively monitor the spread of HPAIVs, especially in areas where migratory bird species congregate.

CONTENT

INTRODUCTION

Influenza

Influenza viruses can cause both annual epidemics and pandemics. Seasonal influenza virus infections in humans cause annual epidemics that result in millions of human infections worldwide and lead to substantial health and economic burdens [1]. Influenza infection is characterized by sudden onset of fever, headache, cough, muscle and joint pain, severe malaise, sore throat and rhinitis and can result in death in juvenile, elderly and chronically diseased [2]. Influenza pandemics can also have devastating effects globally, as some pandemics have resulted in millions of deaths [3]. These pandemics occur when a new strain of the influenza virus is transmitted to humans from another animal species [4].

Avian Influenza viruses

There are three types of influenza viruses that affect people, called Influenza A, B and C. They are members of the *Orthomyxoviridae* family [5]. Influenza A is the most common subtype and responsible for many epidemics and pandemics [4]. Influenza A viruses are negative-sense, single-stranded RNA viruses containing eight different RNA segments. The eight RNA segments of the influenza A virus encode 11 viral proteins. These proteins include the polymerase proteins (PB1, PB2, PA, PB1-F2), nucleocapsid protein, hemagglutinin, neuraminidase, matrix proteins (M1, M2), and nonstructural proteins (NS1, NS2) [5]. Influenza A viruses are divided into subtypes based on two proteins on the surface of the virus: the hemagglutinin (HA) and the neuraminidase (NA). There are 16 different HA subtypes (H1 through H16) and nine different NA subtypes (N1 through N9) [6]. Targeting of HA protein is the main determinant of protective humoral immunity. In addition, the binding affinity of hemagglutinin to the sialic acid residues partly accounts for the host specificity of the various influenza A virus subtypes [5]. In contrast to influenza B and C, influenza A viruses can not only affect humans but can also cause influenza in other mammalian species and in a wide range of avian species. The natural host of influenza A viruses are wild aquatic birds. Avian influenza virus (AIV) infections in birds are mostly low pathogenic (LP), which cause minimal adverse health effects. New AIVs can arise from either point mutations (antigenic drift), recombination of partial genes, or genetic reassortment of whole genes (antigenic shift). Point mutations are responsible for most AIV evolution [7]. In addition, changes in the AIV gene can increase virulence resulting in highly pathogenic (HP) viruses [8]. AIVs are not only subdivided based on their pathogenicity, but also fall into two geographically distinct genetic lineages, the North American and the Eurasian [9]. AIVs are shed in faeces and trough secretions of the oral cavity and respiratory tract [10] leading to transmission. Humans are mostly infected by these AIVs through contact with poultry, which in turn are infected either directly or indirectly by wild birds [11]. The H5 and H7 subtypes are the only viruses known to naturally switch to HPAI upon introduction into poultry and cause human disease and mortality [4,12].

H5 subtype highly pathogenic avian influenza viruses

Since the Asian-lineage subtype H5 highly pathogenic avian influenza virus (HPAIV) was first detected in China in 1996, outbreaks of infection caused by this virus in poultry have been continuous. The H5 HPAIV outbreak resulted in the culling of over 250 million birds worldwide [13] and serious economic losses in the poultry industry [14]. H5 HPAI did not only affect the poultry industry, but was also associated with direct chicken-to-human transmission resulting in a large number of human infections often with fatal outcomes [15]. H5 viruses continue to circulate and evolve into many genetic lineages

and multiple clades [16]. Subclade 2.3.4.4 H5N1 viruses have mixed with several NA subtypes to generate widely circulating H5N2, H5N5, H5N6, and H5N8 subtypes of H5 HPAI viruses [17–19]. In January 2014, novel reassortant HPAI viruses of subtype H5N8, clade 2.3.4.4, were detected in poultry and wild bird carcasses in South Korea [20]. Closely related viruses were also detected in Japan [21] and China [22]. Subsequently, HPAI H5N8 viruses were observed in Europe and North America and were then reintroduced into South Korea and Japan [23]. Genetic analysis showed that this virus was generated by reassortment of HPAI viruses of eastern China. [23].

Scientific problem

No human cases of infection with HPAI H5N8 virus have been reported to date. However, HPAI H5N1 viruses have been associated to human infection [24]. Therefore, the potential public health risk upon human exposure to HPAI H5N8 will be assessed in this review.

ORIGIN OF HIGHLY PATHOGENIC AVIAN INFLUENZA H5N8 VIRUS

The HPAI H5N8 virus was first detected in domestic poultry in China in 2010 [23] and circulated in China until December 2013 [22]. The H5N8 virus was next reported in Korea in a breeder duck farm in Gochang in Jeonbuk province in January 2014. Near Gochang, another infection was reported in broiler ducks in Buan and flocks of Baikal teal carcasses were found in the Donglim reservoir in Gochang [25]. Subsequent outbreaks in Japan, China, Europe, and North America were reported with outbreaks among domestic ducks, chickens, geese, and wild birds [23]. The HPAI H5N8 virus was considered highly pathogenic based on the presence of mutations associated to high pathogenicity [26–28]. This virus was a reassortant virus with the HA gene segment from HPAI H5N1 virus and was categorized as HPAI H5 virus clade 2.3.4.4 [29,30]. Further analysis of the HPAI H5N8 viruses circulating in Korea indicated that the NA genes of these viruses belonged to the N8 subtype of the Eurasian lineage and that they clustered with the H3N8 isolates. Phylogenetic analysis of the six internal genes indicated that H5N8 viruses were reassortant viruses with genes derived from H5N2, H4N2, H11N9 and H5N8 viruses from eastern China [30,31]. Two distinct clusters of HPAI H5N8 viruses were identified: group A (Buan2-like) viruses were detected in China in early 2014 and later in South Korea, Japan, Taiwan, Russia, Canada, the United States, and Europe; group B (Gochang1-like) viruses were detected only in China in 2013 and South Korea in 2014 [32]. Group A H5N8 viruses, representing intercontinental group A (icA), predominated and are further divided into three distinct subgroups, icA1, icA2, and icA3. The icA1 subgroup contains H5N8 viruses from Europe and Russia from late 2014 and three viruses detected in Japan in December 2014. The icA2 subgroup includes H5N8 and, H5 clade 2.3.4.4 North American HPAIV reassortants (H5N2 and H5N1) detected in North America starting in late 2014 and a Japanese virus, A/crane/Kagoshima/KU1/2014 (H5N8) from November 2014. The icA3 subgroup contains H5N8 viruses isolated in Japan in December 2014 and Korea in January 2015 [23]. Co-circulation of group A viruses with low pathogenicity avian influenza (LPAI) viruses led to new reassortants, including H5N1, H5N2, and H5N8 [31]. In 2016, a novel reassortant group B HPAI H5N8 clade 2.3.4.4 virus was detected in a wild bird in Siberia [33], which later spread to Europe [31]. As the viruses isolated in Siberia genetically differed from those found in Europe, the novel HPAI H5N8 clade were subdivided into novel H5N8 Siberia viruses genotype 1 and novel H5N8 European viruses genotype 2 (figure 1) [31]. Thus, although group A viruses were predominant during the initial H5N8 outbreak, currently circulating H5N8 viruses seem to mainly consist of group B viruses.

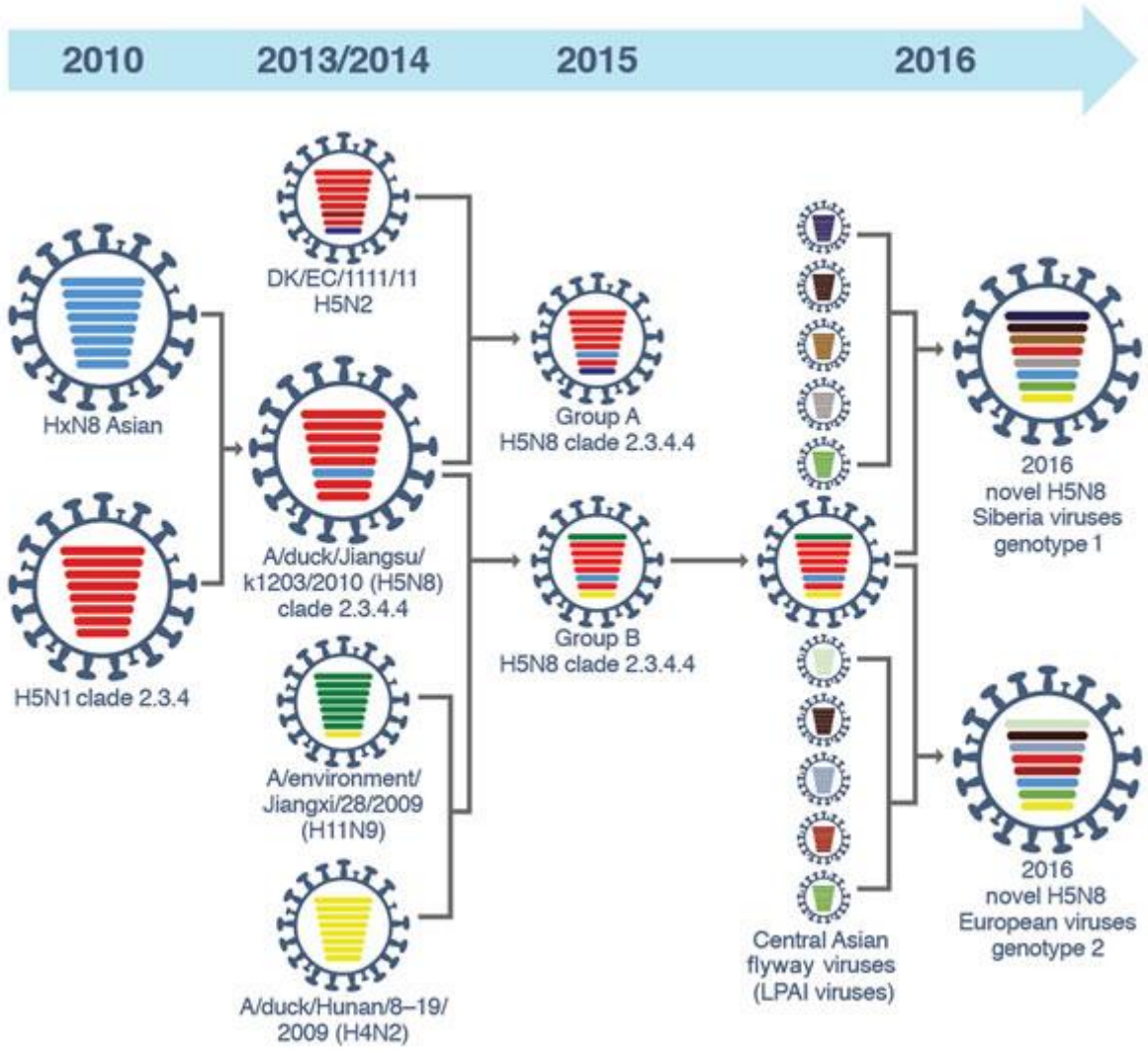

Figure 1. Illustration of original reassortment events of novel highly pathogenic avian influenza (HPAI) A(H5N8) viruses isolated from Siberia and Europe in 2016. The eight gene segments (from top to bottom) in each virus are polymerase basic 2, polymerase basic 1, polymerase acidic, hemagglutinin, nucleoprotein, neuraminidase, matrix, and nonstructural. Each color indicates a separate virus background. In 2010, HPAI A(H5N1) clade 2.3.4 viruses reassorted with subtype N8 viruses from Eurasia and produced A/duck/Jiangsu/k1203/2010(H5N8). Until late 2013, HPAI viruses with H5N8 subtypes circulated in eastern China and South Korea. In 2014, HPAI A(H5N8) viruses reassorted with A/duck/Hunan/8–19/2009(H4N2) and A/environment/Jiangxi/28/2009(H11N9) to generate group B viruses. The subsequent reassortment between HPAI A(H5N8) group B viruses and low pathogenicity (LPAI) viruses circulating along the central Asian flyway led to generation of the novel HPAI A(H5N8) genotype 1 and 2 viruses [31].

SPREAD OF HIGHLY PATHOGENIC AVIAN INFLUENZA H5N8 VIRUS

Virus spread traditionally has been attributed to transport of infected poultry, infected poultry products, or HPAIV-contaminated materials [34]. However, wild aquatic birds are the natural reservoir for AIVs and several observations suggest that wild birds could potentially be involved in the spread of HPAI H5N8 viruses [34].

Introduction H5N8 virus

HPAI H5N8 virus had never previously been detected in Korea in the active surveillance of poultry and wild birds, suggesting the virus was introduced from abroad. In addition, genetic analysis and migration

route tracking of wild birds suggested that the HPAI H5N8 strain discovered in Korea in 2014 likely originates from the novel HPAI H5N8 strain discovered in China [22,32,35] . In line with the hypothesis that wild birds are responsible for the introduction and initial spread of HPAI H5N8 virus is the fact that H5N8 antibodies were first detected in Baikal teal in Korea. Baikal teal are migratory birds, which spend every winter in western habitat sites throughout South Korea [32]. The timing of the HPAI H5N8 virus outbreak in Korea is also in accordance with the migration of these wild birds. In addition, migration of a subpopulation of Baikal teal to other habitat sites in the west of South Korea was reported. The flyways of the Baikal teal flock were similar to the pattern of viral spread between the regions in South Korea, suggesting that Baikal teal participated in the spread of HPAI H5N8 virus between regions during the initial stage of the outbreak [32]. However, HPAI H5N8 virus was isolated from a number of different bird species, including healthy captured mallard, spot-billed duck and common teal, suggesting that these species may also be involved in the introduction and spread of the HPAI H5N8 virus [32]. Thus, the introduction of HPAI H5N8 virus to Korea is likely attributed to migration of wild birds.

Spread H5N8 virus

Spread of HPAI H5N8 virus has also been attributed to wild bird migration. That is, genetic analyses of the HA showed that H5N8 viruses detected in Europe, Russia and in North America belonged to group A H5N8 viruses and were genetically most closely related to H5N8 viruses detected in Japan and South Korea in 2014 [36]. These findings suggest that European and North American H5N8 viruses originate from H5N8 viruses in Asia and could potentially be introduced in Europe and North America by migratory birds. In addition, ring recoveries of migratory duck species from which H5N8 viruses were isolated provide evidence for indirect migratory connections between East Asia and western Europe and between East Asia and North America [36]. Furthermore, most affected poultry farms were located in areas where wild waterfowl are abundant [37,38]. Therefore, contact with infected wild birds or contaminated wild-bird faeces was suggested as the most likely route of virus introduction in the United States, Germany, The Netherlands, United Kingdom and Italy [37]. In addition, South Korea reported the export of a low number of live chickens and no export of live domestic ducks, suggesting that international trade in live poultry unlikely contributed to the long-distance spread of South Korean clade HPAI H5N8 virus [39]. These findings suggest that wild birds have contributed to the spread of HPAI H5N8 viruses, indicating proper surveillance of further spread of these viruses would be difficult.

Common breading grounds and migratory staging grounds, such as Siberia, especially pose a threat in the long-distance spread of HPAIV. The Baikal teal, which as stated previously was one of the most H5N8-infected wild birds, is a migratory bird that over winters in Korea and breeds in North Eastern Siberia during the summer months [40]. The breeding grounds and migratory staging grounds for Baikal teal overlap with those for many other migratory species including mallards, pochards, widgeon, common teal, whooper swans and tundra swans [41]. These common breeding and migratory staging grounds can result in the introduction of HPAI H5N8 virus in large numbers of wild bird species and can play a role in the development of novel virus reassortants. Certain populations of the Mandarin duck, which is a resident bird of South Korea and Japan, also migrate between Siberia and China, suggesting that HPAI-infected Mandarin ducks can also be involved in transmission among countries [42]. Another potentially important staging ground regarding the spread of HPAI H5N8 viruses is the Tanguar haor area in Bangladesh, where approximately 200 migratory bird species overwinter. In addition, Tanguar haor is located in the central Asian flyway and is near the Eastern Asian-Australian and Black Sea-Mediterranean flyways (figure 2) [31], which are flyways associated to HPAI H5N8 infections. These

findings suggest that common breeding and staging grounds, including Siberia and the Tanguar haor area, should be monitored extensively to limit further spread of HPAI H5N8 viruses and to limit the potential development of novel reassortants.

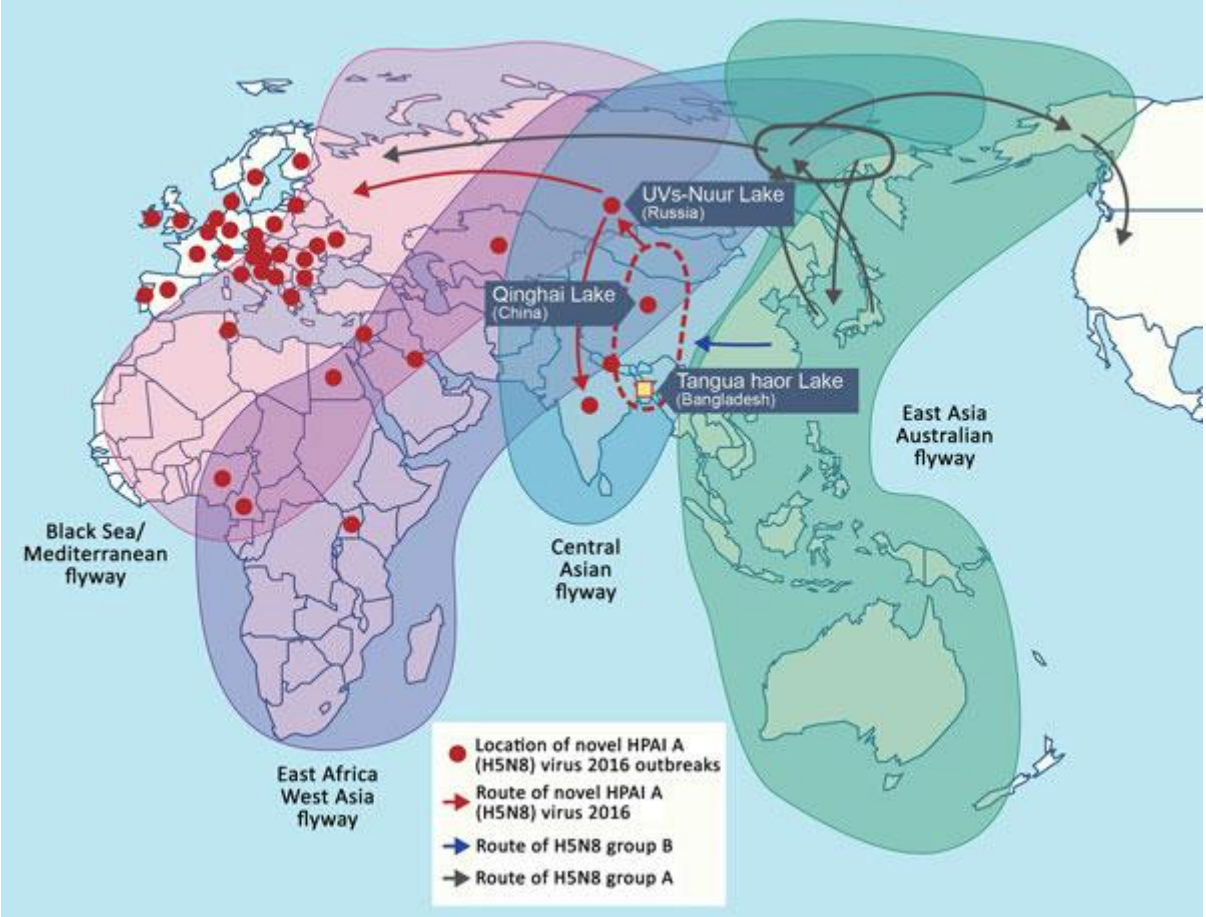

Figure 2. Global movement of wild birds and geographic distribution of novel HPAI A(H5N8) viruses, 2016. Influenza A viruses were isolated from wild birds and free-ranging domestic ducks in the Tanguar haor region of Bangladesh (yellow square) during February 2015-February 2016. Dissemination of novel HPAI A(H5N8) clade 2.3.4.4 viruses (red arrows). Dashed circles indicate location of reassortment between HPAI A(H5N8) group B viruses and low pathogenicity avian influenza viruses circulating along the Central Asian flyway. HPAI: highly pathogenic avian influenza [31].

PATHOGENESIS AND TRANSMISSION OF HIGHLY PATHOGENIC AVIAN INFLUENZA H5N8 VIRUS

Affected population

As mentioned before, HPAI H5N8 infection was confirmed in many wild bird species. Additionally, HPAI H5N8 virus was observed in many poultry farms. The majority of infected poultry species were ducks. Infection was also confirmed in chickens and other kinds of poultry, including mallards, Muscovy ducks, ostriches and geese [17,22,32,43]. In South Korea alone, during January-July 2014, HPAI H5N8 virus was confirmed in 212 poultry farms and in 38 wild birds species [35]. To further explore the potential threat of HPAI H5N8 viruses worldwide, the pathogenesis and transmission of HPAI H5N8 viruses in these avian species will be discussed in the following chapter. The potential of HPAI H5N8 viruses to affect mammals will also be further explored trough evaluation of the pathogenesis and transmission in mammalian species.

Avian species

Aquatic birds

Domestic ducks and geese are regarded as intermediate reservoirs between the aquatic bird and domestic poultry in the influenza ecosystem [44]. In addition, the majority of HPAI H5N8 infections were observed in ducks and geese [35]. Therefore, many studies investigated the pathogenesis and transmission potential of H5N8 viruses in these aquatic bird species. The high number of dead wild birds, mainly Baikal teals, infected with HPAI H5N8 virus found around Donglim Reservoir in southwestern Korea during the HPAI H5N8 outbreak suggests that H5N8 viruses were pathogenic in these ducks [45]. In accordance, domestic ducks naturally infected with HPAI H5N8 virus showed high pathogenicity, neurological signs and increased mortality [46]. In contrast, HPAIV H5N8 has been detected several times in apparently healthy wild water birds, including ducks, in Germany, The Netherlands and Sweden. These ducks showed no clinical signs and no increase in mortality, indicating HPAI H5N8 virus was less virulent in these cases [38]. In experimentally inoculated ducks, no to mild clinical signs or mortality were observed [20,42,47–50] and ducks were shown to be less susceptible to infection and disease than geese [50]. In contrast, another study showed that HPAI H5N8 viruses were highly pathogenic in inoculated mallard ducks often with a fatal outcome [49]. Regarding viral shedding and transmission, HPAI H5N8 virus successfully replicated in inoculated birds and were successfully transmitted to co-housed birds (figure 3B and D) [20,42,47–50]. In addition, viral shedding and replication in tissues were higher in ducks infected with H5N8 than in those infected with H5N1 viruses [20,42]. It was also indicated that viral shedding in inoculated and contact-exposed ducks was more prevalent in oropharynx than the cloaca (figure 3) [42,50], suggesting transmission was more likely to occur through the respiratory tract than through the digestive tract. The contradicting findings regarding pathogenesis of HPAI H5N8 viruses in ducks might result from the various virus strains used in the different studies. That is, ducks showing moderate pathogenicity were mostly inoculated with group A H5N8 viruses [42,47,48], while ducks showing high pathogenicity were inoculated with group B viruses [49], suggesting some virus strains are better adapted to ducks. Additionally, virulence in ducks could vary between species. This is in accordance with the study that showed that mortality rates of domestic ducks and mallard ducks intranasally inoculated with the H5N8 virus were 0–20%, and 0%, respectively [20]. Thus, most HPAI H5N8 viruses have relatively low pathogenicity in ducks, but certain HPAI H5N8 virus strains seem to be better adapted to a certain duck species resulting in increased pathogenicity.

Chickens and pigeons

As the chicken is one of the most common and widespread domestic animals [51], infection with influenza in chickens can have detrimental consequences. Therefore, a number of studies investigated the effect of HPAI H5N8 infection in chickens. HPAI H5N8 viruses were shown to be highly pathogenic in chickens and HPAI H5N8 infection in chickens resulted in increased mortality (figure 4) [27,48,49,43,52]. In contrast, a study by Bertran and colleagues showed that HPAI H5N8 virus was only moderately pathogenic in chickens. However, when a virus dose similar to the dose used in the other studies, 100% mortality was observed [53]. Virus transmission was also determined. HPAI H5N8 viruses were detected from both oropharyngeal and cloacal swab samples and were successfully transmitted to co-housed birds [52]. However, transmissibility was lower compared to HPAI H5N1 virus [52,53]. Clinical lesions observed upon H5N8 infection were consistent with lesions expected with a HPAIV infection, including hemorrhages in the legs, comb and wattle, and petechial hemorrhages and necrosis in several different

organs [37,53]. These results suggest that HPAI H5N8 viruses are highly pathogenic in chickens. However, HPAI H5N8 viruses do not seem to be easily transmitted to contact chickens and the rate of chicken-to-chicken transmissibility might vary between HPAI H5N8 strains. Thus, HPAI H5N8 viruses are highly pathogenic in chickens, but might not be fully adapted to chickens. However HPAI H5N8 viruses have previously been shown to cause high mortality in chicken farms, demonstrating the disastrous consequences HPAI H5N8 viruses can cause in domestic chickens [27].

Because pigeons inhabit a wide geographic region, infection and replication of HPAI H5N8 in this species can be a threat to the poultry industry and public health [42]. Transmission of the HPAI H5N8 viruses was not observed in pigeons, although virus was detected from both oral and cloacal swab samples. In addition, frequency and severity of the viral infection was relatively milder in pigeons than in ducks [42]. Thus, HPAI H5N8 viruses do not seem to pose a threat to the poultry industry trough infection in pigeons.

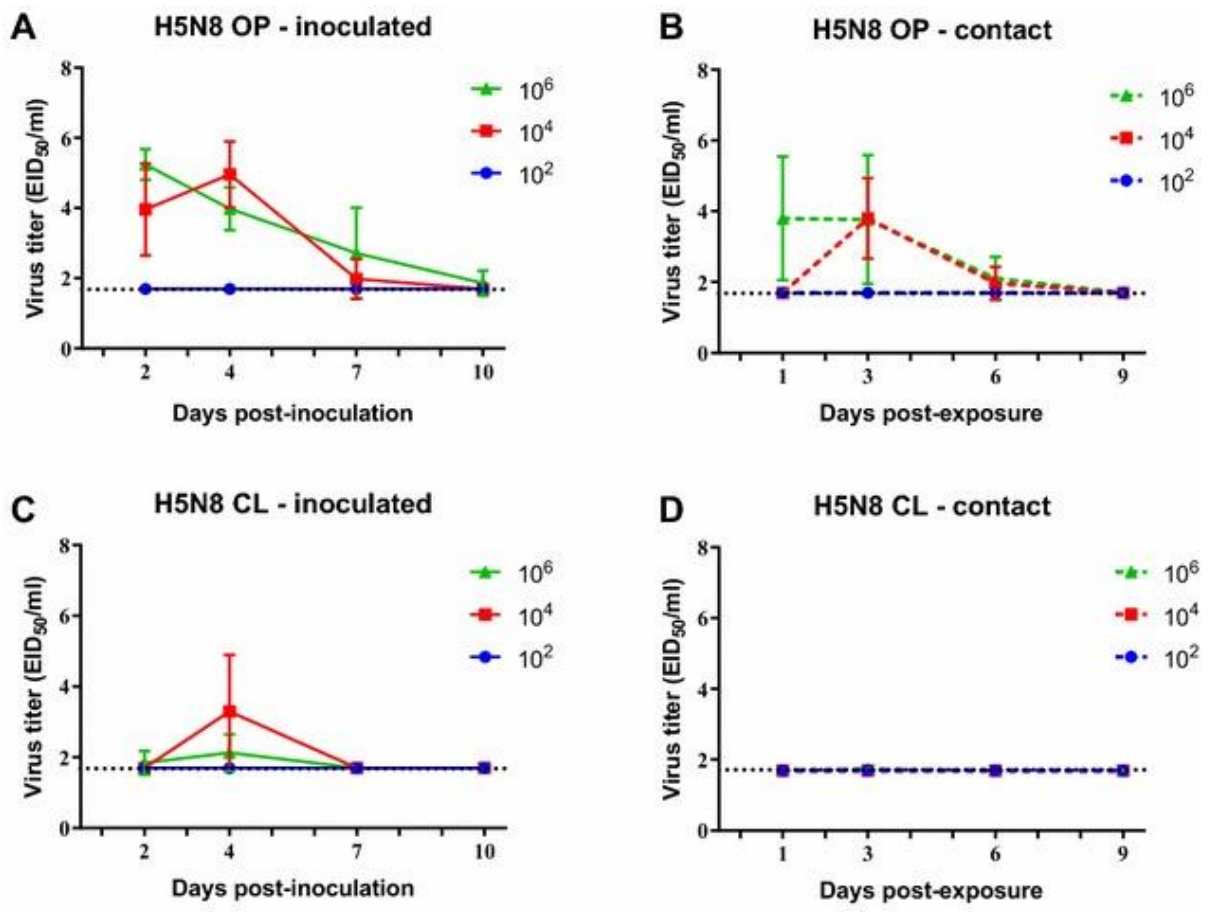

Figure 3. Mean oropharyngeal (OP) and cloacal (CL) viral shed from 2-week-old Pekin ducks directly inoculated (A and C) or contact-exposed (B and D) with H5N8 HPAI viruses. Ducks were inoculated with 10^2, 10^4 and 10^6 EID_{50} of H5N8 virus or contact-exposed to inoculated ducks one day post-inoculation. Virus titers were determined by qRRT-PCR. Bars represent the standard deviation of the mean. Swabs from which virus was not detected were given a numeric value of $10^{1.7}$ EID_{50}/mL (adapted from [50].

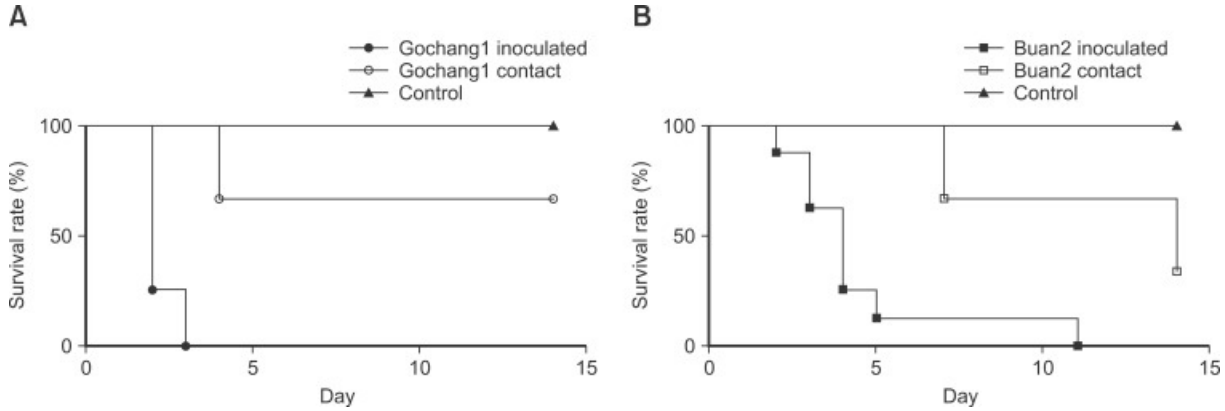

Figure 4. Survival curves for the experimentally inoculated and contact chickens. Eight 5-week-old chickens were inoculated with $10^{6.5}$ EID_{50}/0.1 mL of A/breeder duck/korea/Gochang 1/2014 (H5N8) (Gochang1; A) or A/broiler duck/korea/Buan2/2014 (H5N8) (Buan2; B). When comparing the survival curves of the two viruses, mortality rates were 100% for the inoculated chickens. There were significant differences between the survival curves of the two viruses for the contact chickens (log-rank test, $p < 0.05$) [52].

Mammalian species

Mice

To further explore the potential risk HPAI H5N8 viruses pose to humans, pathogenesis in various mammal models was assessed. In mice, virulence varied from moderate [48,49,54,55] to high [22,49] depending on the HPAI H5N8 virus strain (figure 5) [49]. Inoculation of mice with HPAI H5N8 was associated with increased mortality, morbidity and successful virus replication [49]. Kim and colleagues also showed that H5N8 virus is less pathogenic in mice than H5N1 viruses as measured by antigen detection, virus replication and shedding and cytokine expression [48]. A study by Choi and colleagues showed a dramatic increase in morbidity in mice infected with H5N8 viruses during lung-to-lung sequential passages. However, HPAI H5N8 was shown to be moderately pathogenic in the initially inoculated mice [56]. The difference in pathogenicity of HPAI H5N8 viruses in mice might result from the various virus strains used in the different studies. That is, the viruses used in the studies indicating HPAI H5N8 viruses were highly pathogenic in mice originated from group B HPAI H5N8 viruses and shared amino acid substitutions [22,49] associated with pathogenicity in mice [57], while the studies indicating HPAI H5N8 viruses were moderately pathogenic in mice originated from group A viruses [48,54,55]. Thus, HPAI H5N8 viruses varied from moderate to high pathogenicity in mice, with group B viruses being highly pathogenic and group A viruses being moderately pathogenic.

Ferrets

As ferrets show similar susceptibility to infection with human and avian influenza viruses and develop respiratory disease similar to that observed in humans, ferrets have been used in influenza research since 1933 [58]. Therefore, a number of studies also tested pathogenicity of HPAI H5N8 viruses in a ferret model. Group A and B HPAI H5N8 viruses both showed poor replication and mild virulence in ferrets and were not transmitted to contact ferrets (figure 6) [48,54,55,59,60]. The HPAI H5N8 virus was also shown to be less pathogenic in ferrets than HPAI H5N1 virus [60]. In addition, mutations in the HPAI H5N8 virus strains were suggested to cause the impaired replication and inefficient contact transmission among ferrets [48,61]. In contrast, it was indicated that HPAI H5N8 viruses can acquire a mutation in the PB2 region (the $PB2_{701N}$ substitution) associated with mammalian host-adaptation and virulence during

replication in ferrets [48]. These findings suggest that HPAI H5N8 viruses are not fully adapted to ferrets, but might be able to become more adapted through genetic changes once an infection in a ferret occurs.

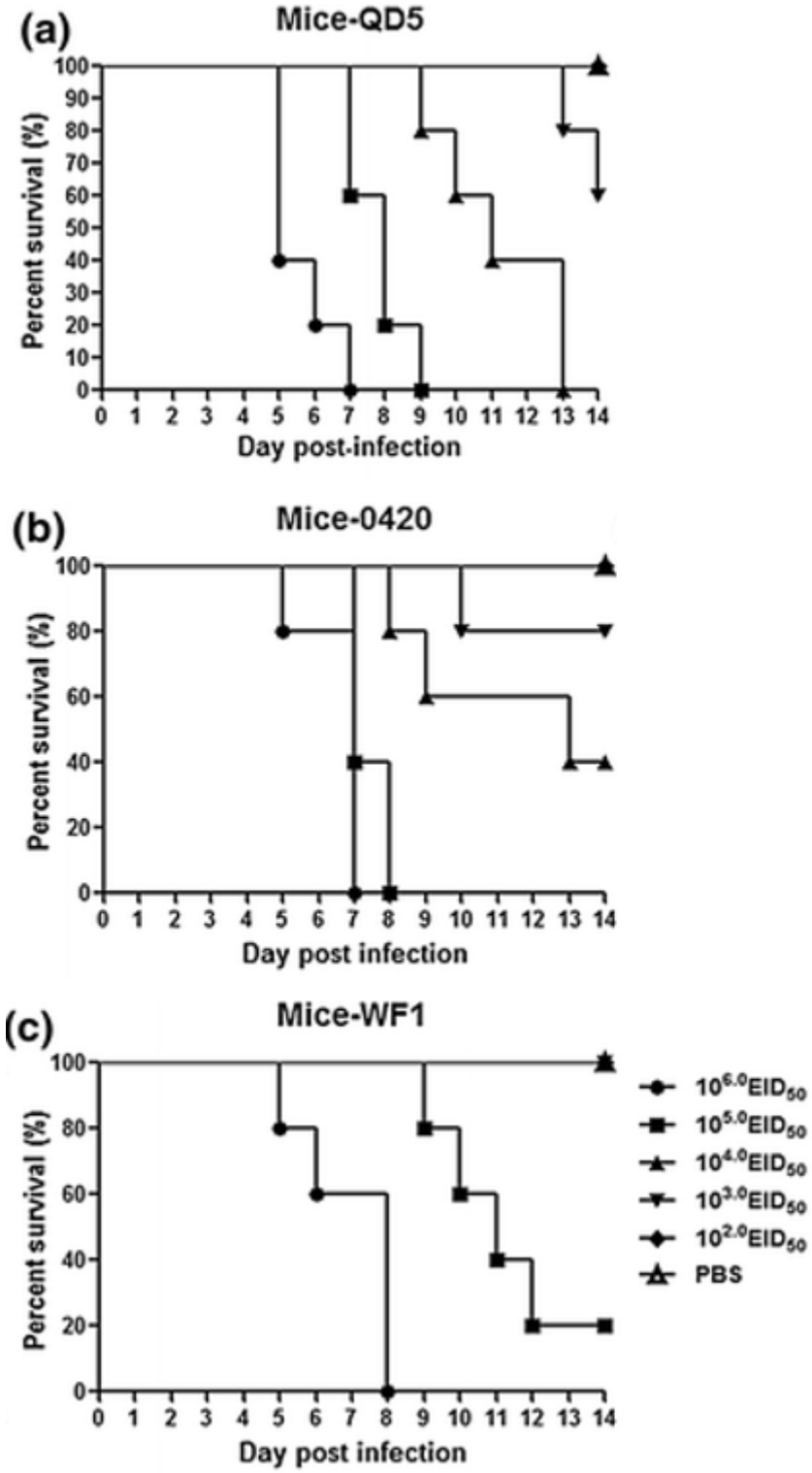

Figure 5. Pathogenicity of the three H5N8 viruses in mice. Five six-week-old mice were inoculated intranasally with $10^{2.0}$ to $10^{6.0}$ EID_{50} of A/goose/Jiangsu/QD5/2014 (QD5), A/goose/Shandong/WFSG1/2014 (WF1) or A/goose/Yangzhou/0420/2014 (0420) H5N8 viruses. Mortality caused by the QD5 (A), 0420 (B) and WF1 (C) H5N8 viruses was determined based on the survival rate(adapted from Li et al., 2016) [49].

Cats and dogs

Virulence of HPAI H5N8 virus was also tested in cats and dogs. HPAI H5N8 virus replicated successfully in respiratory tissues and was moderately pathogenic in both cats and dogs, with cats being more susceptible to H5N8 infection than dogs. However, transmission to co-housed animals was not observed [48]. These findings suggest that HPAI H5N8 viruses are not fully adapted to cats and dogs.

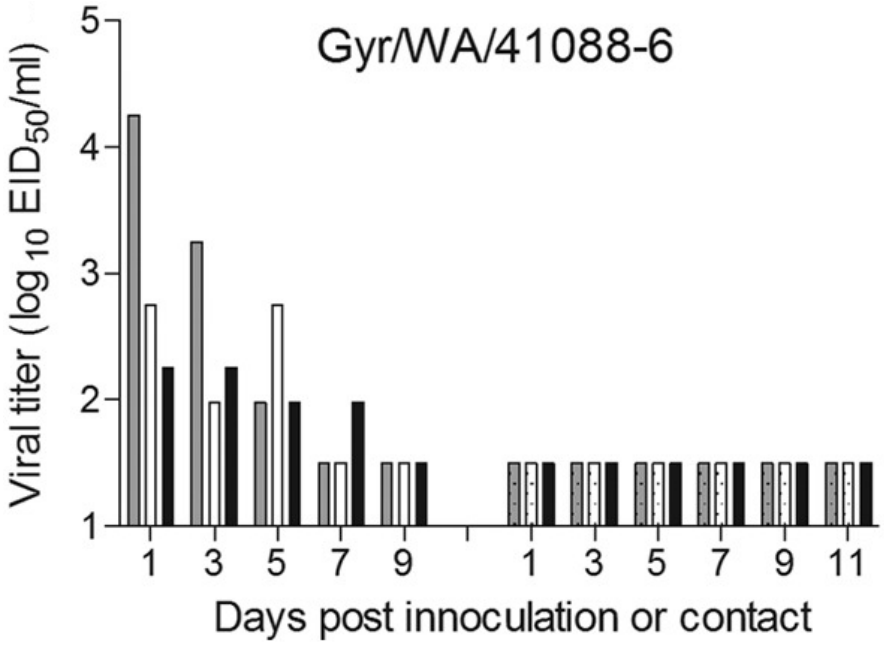

Figure 6. Transmissibility of H5N8 influenza virus in ferrets. Groups of three 6-month-old ferrets were inoculated intranasally with 10^6 EID_{50} H5N8 (Gyr/WA/41088-6) virus. The following day, a serologically naive ferret was placed in the same cage with each inoculated ferret for the assessment of virus transmission between ferrets in direct contact. Nasal washes were collected from each ferret on the indicated days postinoculation or after contact. The results from individual ferrets are presented. The virus titers are presented as $\log_{10}$ EID_{50}/ml. The limit of detection is 1.5 $\log_{10}$ EID_{50}/ml (adapted from Kaplan et al., 2016) [54].

BIOLOGICAL PROPERTIES OF HIGHLY PATHOGENIC AVIAN INFLUENZA H5N8 VIRUS

Properties of the HA protein

Receptor specificity

The HA protein of influenza A viruses is known to influence virus replication, transmission and pathogenicity and plays a central role in the host range restriction of influenza viruses [55]. To further explore the potential of HPAI H5N8 viruses to affect humans, it is therefore of interest to look into the biological properties of the HA protein. One of the HA properties known to contribute to host range, pathogenicity, and transmissibility in avian and mammalian species is receptor specificity [62]. The HA protein of human influenza viruses preferentially recognize α-2,6-linked sialic acids (human-like receptor), whereas the HA protein of avian influenza viruses preferentially recognize α-2,3-linked sialic acids (avian-like receptor) [62]. Although HPAI H1N1 viruses had a strong preference for human-like receptors (figure 7A) [22], HPAI H5N8 viruses had a strong preference for avian-like α-2,3-linked sialic acids (figure 7B) [22,48,49,55]. However, they also recognized human-like α-2,6-linked sialic acids (figure

7B) [22,48,49,55], with considerably higher affinity than HPAI H5N1 viruses [49]. The HA protein of HPAI H5N8 viruses was shown to contain amino acid substitutions [37], which are associated with avian receptor specificity [63] and did not contain the E627K or D701N substitutions [22,30], which are commonly associated with the adaptation of HPAI H5N1 viruses to mammalian hosts [64,65]. In contrast, the HA protein of HPAI H5N8 viruses was also shown to contain amino acid substitutions [37,46,61], which have been previously reported to be associated with enhanced human receptor specificity [66]. These findings together indicate that the HA of most HPAI H5N8 viruses bind weakly to the dominant receptor of the human upper respiratory tract, which is a barrier to human infection. However, certain HPAI H5N8 viruses bound better to human-like receptors than the human-adapted HPAI H5N1 virus and certain HPAI H5N8 viruses contained mutations associated to mammalian adaptation, indicating there might be some mutated HPAI H5N8 virus strains that are already partially adapted to mammals.

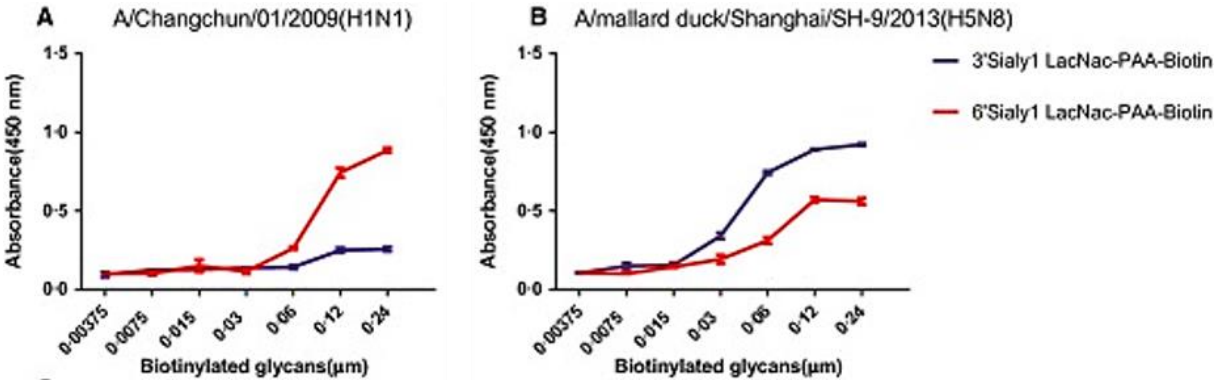

Figure 7. Receptor-binding properties of the novel H5N8 SH-9 virus. Receptor-binding specificities of a human H1N1 virus (A/Changchun/01/2009(H1N1) (A) and the novel SH-9 virus (A/mallard duck/Shanghai/SH-9/2013 (H5N8) (B) were evaluated using a biotinylated α-2,3 glycan (blue line) and an α-2,6 glycan (red line)(adapted from Fan et al., 2014) [22].

Cleavability

The cleavability of the HA protein is another major determinant for IAV pathogenicity. HPAIVs possess a unique multibasic cleavage site in the HA protein that allows cleavage of the HA0 precursor without extracellular protease [67]. HA is initially synthesized as an HA0 precursor that is subsequently cleaved into the two functional proteins HA1 and HA2. This cleavage step is essential for virus infectivity since uncleaved HA is able to mediate virus attachment but is unable to mediate the fusion step necessary for the initiation of infection [68]. The HA proteins of H5N8 viruses contained a multibasic cleavage site associated to HPAIVs. However, the observed cleavage site differed from those found in HPAIVs adapted for infection and transmission in mammals [55], again suggesting the HPAI H5N8 viruses are not yet fully adapted to mammals.

pH sensitivity

Another factor contributing to host range, pathogenicity, and transmissibility in avian and mammalian species is pH sensitivity of acid-triggered membrane fusion. A low pH of HA activation is required for human infection [69–72]. HPAI H5N8 viruses had a high activation pH [55], suggesting that the fusion properties of the HA protein of HPAI H5N8 viruses are also poorly adapted to mammals.

Biological properties

To further explore the potential of HPAI H5N8 viruses to affect humans, the biological properties of the H5N8 virus was determined using human cell lines and tissue. HPAI H5N8 viruses replicated in human nasal respiratory epithelium and lung tissues as well as HPAI H5N1 viruses. In addition, cells infected with H5N8 viruses were able to attach to human respiratory tissues, although this attachment was not as extensive as that exhibited by H1N1 virus [48]. Another study showed that three different HPAI H5N8 viruses were able to replicate in A549, a human lung carcinoma cell line [49]. However, when replication of HPAI H5N8 viruses was tested in a human bronchial epithelium cell line at a temperature of 33 °C, the natural temperature of the mammalian upper airway, instead of at a temperature of 35 °C [48] or 37 °C [54], replication was shown to be insufficient (figure 8) [54]. As transmission via respiratory droplets and aerosols is the main route for influenza virus transmission between humans, the ability to replicate in the mammalian airway is an important barrier to cross to allow human infections. This suggest that replication of HPAI H5N8 viruses is not fully adapted to the mammalian upper airway.

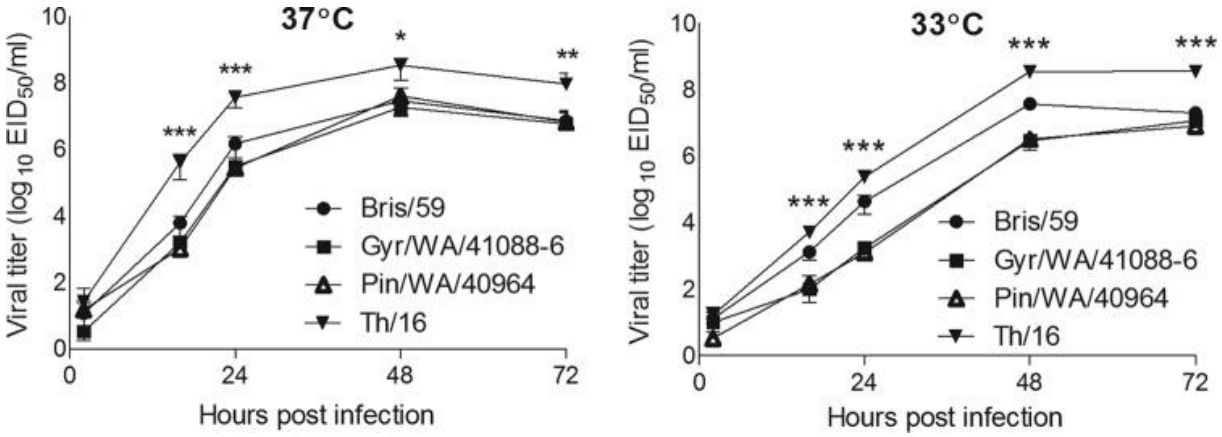

Figure 8. Replication kinetics of influenza viruses in polarized human airway epithelial cells. Calu-3 cells grown on transwells were infected apically in triplicate at a multiplicity of infection of 0.01 with Pin/WA/40964 (H5N2), Gyr/WA/41088-6 (H5N8), Bris/59 (H1N1), or Th/16 (H5N1). The cells were incubated at 37°C (A) or 33°C (B), and culture supernatants were collected at 2, 16, 24, 48, and 72 hours post-inoculation for virus titer determination by standard plaque assay. Asterisks indicate the statistical significance between Th/16 and other tested H5Nx viruses (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) [54].

PREVENTION AND TREATMENT OF HIGHLY PATHOGENIC AVIAN INFLUENZA H5N8

Prevention influenza infections

The main option to prevent influenza virus infections is vaccination. Effectiveness against HPAI H5N8 viruses of available vaccines has therefore been evaluated. Various pre-pandemic H5 vaccine strains did not elicit antibodies that cross-reacted with the H5N8 HA, suggesting that these selected pre-pandemic H5 vaccine strains did not match antigenically [60]. However, certain pre-pandemic vaccines against previous HPAI H5N1 strains did provide sufficient protection against the recent heterologous HPAI H5N8 virus in mice and ferrets, with the clade 2.5 strain showing the highest cross-reactivity and protection in mice and ferrets [73]. Another vaccine prepared from a H5N1 strain was also effective against the H5N8 HPAI virus in chickens. Chickens were completely protected from disease manifestations and death and did not shed any virus [74]. A vaccine based on recombinant virus-like particles (VLPs), containing H5 and N1 genes, protected chickens against H5N1 viruses, but also against H5N8 viruses (figure 9) [75]. Clade 2.3.2 and 2.3.2.1 HPAI H5 vaccines were also shown to protect chickens

against HPAI H5N8 virus infection, although virus shedding was still observed until 7 days post challenge [76]. Additionally, protection against HPAI H5N8 virus infection in chickens was most optimal when using antigenically matching vaccines [77]. These findings suggest that there are potential vaccines available today that will be able to protect poultry against HPAI H5N8 infection. However, repeated use of pre-pandemic vaccines leads to antigenic drift in AIVs due to the presence of immune pressure. AIVs could therefore escape from vaccine protection [74]. In addition, protection was most optimal using vaccines matching the infectious virus. It is therefore essential to develop future vaccines that match currently circulating HPAI H5N8 viruses.

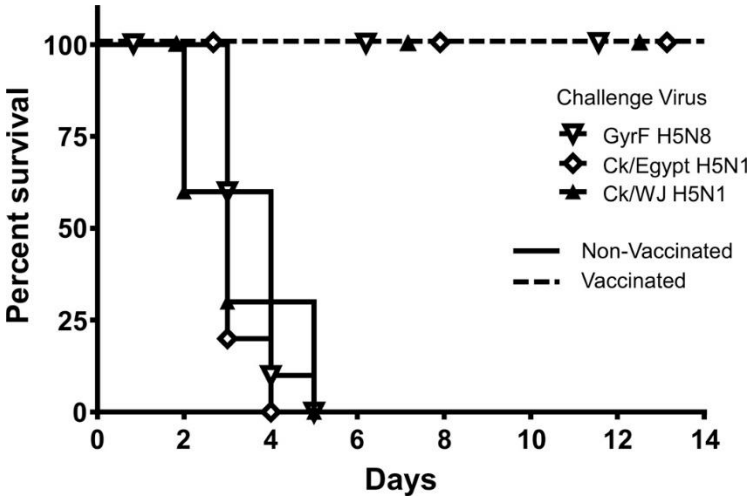

Figure 9. Kaplan-Meier survival plots for protection of triple-clade H555 VLP vaccinated chickens against HPAI H5N1 or H5N8 challenge. Chickens were unvaccinated (Sham, solid line) or vaccinated with triple-clade H555 VLP at one day-of-age and 21 days of age (dashed line), then randomly split into three groups, and each group was challenged (10^6 EID_{50} per bird) at 35 days of age with one of the indicated HPAI H5 isolates: A/Gryfalcon/Washington/2014 H5N8 (clade 2.3.4.4), A/Ck/WJSubang/2007 H5N1 (clade 2.1.3) or A/Ck/Egypt/2010 H5N1 (clade 2.2.1). VLP: recombinant virus-like particle [75].

Treatment influenza infections

The main option to treat influenza virus infections is antiretroviral therapy. The neuraminidase inhibitors (NAIs) are the only class of antiviral drugs currently recommended for the treatment of influenza virus infections. However, NAI-resistant viruses can emerge either during drug treatment or naturally during virus evolution [78]. Therefore, a number of studies have investigated the susceptibility of HPAI H5N8 viruses to these drugs. HPAI H5N8 viruses were shown to be susceptible to three different NAIs: oseltamivir, zanamivir, and peramivir [37,55]. However, there was one study that showed that the NA of HPAI H5N8 viruses contained the I314V substitution, which is a molecular marker for oseltamivir resistance [30]. Additionally, a mutation in the M2 protein of H5N8 viruses, related to resistance to adamantanes [79], was detected [28,30,37,48,55,61], suggesting that these viruses are resistant to M2 ion channel blockers. Taken together, these data suggest that NAIs, but not M2 ion channel blockers, will be a viable treatment option for the infections caused by HPAI H5N8 viruses.

CONCLUSIONS

Collectively, HPAI H5N8 viruses are better adapted to avian species than mammalian species. That is, although HPAI H5N8 viruses were capable of infecting both avian and mammalian species, horizontal transmission was more effective in avian species. In addition, HPAI H5N8 viruses preferentially bound avian-like receptors and replication in a human bronchial epithelium cells line was unsuccessful under conditions similar to those in mammals. However, the tendency of HPAIVs to mutate and reassort might make viruses more adapted to mammalian species. This hypothesis is supported by the finding that group B HPAI H5N8 viruses seem better adapted to mammals than group A HPAI H5N8 viruses. As the currently circulating H5N8 viruses mainly consist of group B viruses, this could increase the prospect of human infection. Moreover, HPAI H5N8 virus continue to circulate in wild birds and cause rapid geographical spread, further increasing the likelihood of human exposure. Many avian species sharing common breeding and staging grounds were shown susceptible to HPAIV infection, indicating that reassortment of H5N8 viruses with other influenza viruses can occur in these areas. The tendency of HPAI viruses to mutate and reassort has already been shown by the high number of HPAI virus strains currently circulating worldwide. Moreover, the potential of HPAIVs to affect humans has previously become evident by detection of human infections caused by the zoonotic HPAI H5N1 viruses. This is especially of interest since the HPAI H5N8 virus are genetically related to HPAI H5N1 viruses. The tendency of HPAIVs to mutate might also pose a risk regarding the treatment of the virus. That is, while currently circulating HPAI H5N8 viruses were shown to be susceptible to NAIs and certain vaccines were shown to protect against H5N8 infection, susceptibility to treatment can be altered when mutations occur.

These results together suggest that the public health threat of the HPAI H5N8 strains is low. However, the rapid geographical spread of HPAI H5N8 viruses, their ability to infect various avian and mammalian species without causing clinical signs and the tendency of influenza A viruses to mutate and reassort are major concerns. Therefore, it seems of interest to extensively monitor the spread of HPAIVs, especially in areas where migratory bird species congregate.

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