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H9 Influenza Viruses: An Emerging Challenge

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Abstract

Influenza Aviruses (IAVs) of the H9 subtype are enzootic in Asia, the Middle East, and parts of North and Central Africa, where they cause significant economic losses to the poultry industry. Of note, some strains of H9N2 viruses have been linked to zoonotic episodes of mild respiratory diseases. Because of the threat posed by H9N2 viruses to poultry and human health, these viruses are considered of pandemic concern by the World Health Organization (WHO). H9N2 IAVs continue to diversify into multiple antigenically and phylogenetically distinct lineages that can further promote the emergence of strains with pandemic potential. Somewhat neglected compared with the H5 and H7 subtypes, there are numerous indicators that H9N2 viruses could be involved directly or indirectly in the emergence of the next influenza pandemic. The goal of this work is to discuss the state of knowledge on H9N2 IAVs and to provide an update on the contemporary global situation.

Influenza A viruses (IAVs) of the H9 subtype are widespread in nature in many species of wild waterfowl and shorebirds worldwide. The H9 subtype IAVs are among the non-notifiable IAV subtypes identified by the World Animal Health Organization. The literature refers to outbreaks caused by H9 IAVs as low pathogenic avian influenza virus (LPAIV) outbreaks. To our knowledge, there is no natural isolate of H9 IAV associated with a highly pathogenic avian influenza (HPAI) outbreak. More intense and geographically expanded surveillance efforts have greatly improved the wealth of information regarding H9N2 IAVs. Nevertheless, there are still major gaps in our understanding of the global distribution of the H9 IAVs. Herein, we review the current knowledge on the geographic distribution of H9N2 IAVs, as well as their phylogenetic evolution and classification, host range, tropism, pathogenesis, and the risk they pose to public health.

NATURAL HISTORY AND THE ROLE OF POULTRY

Like all other IAV subtypes, the natural reservoir of the H9 subtype IAVs are thought to be the waterfowl and shorebirds of the world (Alexander 2000, 2007; Halvorson 2008). IAV strains of the H9 subtype have been associated with every one of the known nine neuraminidase (NA) subtypes described (Table 1). Interestingly, of the ~9500 unique H9 hemagglutinin (HA) sequences publicly available, ~7200 (>75%) are paired with N2 NA subtype sequences, suggesting preferred association and coevolution of these two gene

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segments in nature. The vast majority of H9 HA sequences correspond to isolates from Asia (~6600 from avian species and ~200 from other hosts). However, the H9 subtype was first discovered in North America, associated with an LPAI outbreak in turkeys in February 1966 in northern Wisconsin in the United States (Smithies et al. 1969; Homme and Easterday 1970). Details of this and other LPAI H9 outbreaks in poultry in the United States, particularly in the main turkey-production states of Minnesota and Wisconsin, have been extensively covered elsewhere (Halvorson et al. 1983, 1997; Halvorson 2009; Perez and de Wit 2016).

The H9 IAVs of Asia have caused significant disease outbreaks in chickens and other land-based poultry, such as quail, pheasant, partridge, and other minor domestic poultry species. These viruses remain enzootic in poultry and are vastly distributed in great parts of Asia, the Middle East, and parts of Africa (Guan et al. 1999; Guo et al. 2000; Liu et al. 2003b). Phylogenetic analysis of the Asian H9N2 viruses suggests that they transmitted multiple times from aquatic birds to poultry (Perez et al. 2003); however, the natural avian reservoir in Asia has not been identified. H9N2 viruses were initially detected in apparently healthy domestic ducks in live poultry markets and farms in Hong Kong from 1975 to 1985 (Markwell and Shortridge 1982; Shortridge 1992). The first evidence of H9 viruses in land-based poultry in Asia was in 1988, when three H9N2 IAVs were isolated from dead quail during an outbreak of respiratory disease that lasted ~3 months in one farm in Hong Kong (Perez et al. 2003). The available data indicate the presence of H9 IAVs in chickens in mainland China in Guangdong province from November 1992 to May 1994 that affected 17 chicken farms and two rare bird farms (Guan et al. 1999). By the late 1990s, H9N2 viruses were detected in most provinces in China, associated with live bird market activity throughout the year with an incidence that ranged from 0.2% to almost 5% depending on the market and time of the year (Liu et al. 2003a; Choi et al. 2004; Li et al. 2005; Lu et al. 2005; Xu et al. 2007a,b). Control measures that have included banning minor poultry and/or mandatory monthly rest days led to decreased circulation, but not eradication of H9N2 IAVs in live bird markets in Hong Kong (Guan et al. 2000; Leung et al. 2012).

PHYLOGEOGRAPHY AND LINEAGE CLASSIFICATIONS

H9 IAVs are the most widespread LPAIVs in poultry in the world (Fig. 1). Two distinct phylogeographic lineages of H9N2 have been described: the American and Eurasian lineages (Fig. 2). From these two major lineages, further clusters can be identified with a complex evolutionary history (Fig. 3). A standardized lineage/clade nomenclature system for H9 viruses is still lacking, although several investigators have proposed systems similar to the WHO/OIE/FAO’s H5 nomenclature system (Chen et al. 2009; Liu et al. 2009; Xu et al. 2018). This is particularly important because H9 IAVs are not typically subjected to the same control mechanisms of stamping out as H5 and H7 subtype IAVs. The numbering system proposed by Liu et al. (2009) and Chen et al. (2009) contemplates H9 IAV lineages on a global scale and places the H9 HA segment into four major sublineages (h9.1–h9.4), which are further structured into clades and subclades (Figs. 2 and 3).

American Lineage

H9 IAVs in North America (h9.1) are predominantly confined to wild birds, especially sea birds, with only occasional self-limiting spill-over events to poultry (most commonly in turkeys) (Table 2; Fig. 3; Morales et al. 2009). Since 2001, there have been no detections of the H9 IAVs in poultry in North America, despite frequent isolations from wildlife during routine surveillance and sporadic occurrences of other LPAI viruses in poultry. At least one vaccine with an H9N2 virus of the North American lineage (A/turkey/Wisconsin/1/1966) was used in China and seems to have been detected in chickens and in plateau pikas *(Ochotona curzoniae)* in China’s Qinghai lake area (Yan et al. 2017; Perez et al. 2019). The long-term impact of such an event in the ecology and epidemiology of H9 IAVs in Asia remains to be seen.

Most of the avian-origin IAV data in South America come from two major surveillance efforts established about 10 years ago in Chile and Argentina, intermittent surveillance in Peru and Brazil, and sporadic surveillance efforts in other parts of South America (Spackman et al. 2006; Ghersi et al. 2009, 2011; Mathieu et al. 2015, 2019; Nelson et al. 2016; Jiménez-Bluhm et al. 2018). South American strains of H9N2 are few and have been isolated from two rosy-billed pochards and one ringed teal in Argentina, and from two mallards, one American oystercatcher, one gray plover, one brown-hooded gull, and one environmental sample in Chile. Serological evidence of H9N2 infections in chickens in Colombia was reported in 2005; however, no virus was isolated and no reports have been made since (Senne 2007). The South American H9N2 HA sequences (clade h9.1.2) are closely related to the North American lineage but show signs of an independent evolutionary trajectory (Figs. 2 and 3; Xu et al. 2012).

Eurasian Lineage

The Eurasian H9 HA sequences fall into three major sublineages: h9.2, h9.3, and h9.4. The emergence of the poultry-adapted H9 IAVs in China has promoted great phylogenetic diversity giving rise to many clades/subclades (Guo et al. 2000; Peacock et al. 2019). The most prevalent clades are named after their prototypic viruses: Y439-h9.2 (A/duck/Hong Kong/Y439/1997), BJ94-h9.3 (A/chicken/Beijing/1/94), and G1-h9.4 (A/quail/Hong Kong/G1/1997). H9 viruses in the Y439-h9.2 are the closest to the Eurasian common ancestor and have been found in wild bird species in Europe, Asia, and Africa with occasional spillover to poultry. The BJ94-h9.3- and G1-h9.4-like viruses circulate mostly in commercial poultry and live bird markets with occasional spillover to wild animals.

Viruses circulating in the wild bird reservoir in Eurasia vary by geographic location but, generally, fall into the Y439-h9.2 lineage (also referred to as Korean lineage) (Figs. 2 and 3; Dalby and Iqbal 2014; Peacock et al. 2019). Y439-like viruses were initially identified from disease outbreaks in chickens in South Korea and from domestic ducks in Hong Kong in the mid-1990s. Since then, Y439-h9.2-like viruses have been isolated sporadically from wild birds and have been associated with outbreaks in domestic birds in Europe, Indonesia, Southeast Asia, Southeast Russia, Oceania, South Africa, Zambia, and Malawi (Abolnik et al. 2006; Bonfante et al. 2018; Peacock et al. 2019). In Europe, vaccination has been adopted to prevent reoccurrences especially in turkeys in Germany and Italy (Capua and Alexander

2009). Meanwhile, the indigenous Korean H9 IAVs have established their own distinct clade/subclades (Korea-restricted, subclades A h9.2.2.3 and B h9.2.2.4) (Fig. 4; Lee et al. 2014, 2016a).

Retrospectively, mismatched vaccination with an outdated Korean subclade A h9.2.2.3 H9N2 virus (Fig. 4) facilitated the emergence of viruses from Korean subclade B h9.2.2.4, which now are the most prevalent in South Korea (Fig. 4; Lee et al. 2016a). Recently, one isolate of an H9 related to the Korean subclade A h9.2.2.2 was reported in poultry from North Korea (Zhang et al. 2017b). Additional studies suggest serological evidence of H9N2 IAVs in poultry in Romania, Nigeria, Laos, and Thailand, but lineage characterization is lacking (Sonnberg et al. 2012; Oluwayelu et al. 2017; Coman et al. 2018; Karlsson et al. 2019).

H9 viruses of the BJ94-h9.3 sublineage, also known as Y280 (A/duck/Hong Kong/Y280/97) or G9 (A/chicken/Hong Kong/G9/97) are found mostly in poultry in China and Southeast Asia (Vietnam, Cambodia, Myanmar, and Indonesia) (Table 2). The BJ94 sublineage viruses have continued to evolve in China with strains distributed among 12 different clades (although some old clades may have been replaced by newer clades). In Japan, these viruses have been reported from illegally imported poultry products (Shibata et al. 2018) and in live poultry and wildlife in the northern Japanese islands (Okamatsu 2003; Abao et al. 2013). The presence of the BJ94-like H9 strains isolated from poultry and wild birds in Eastern Russia (Marchenko et al. 2019) raises questions regarding the geographic distribution and transmission routes of these viruses.

The G1-h9.4 strains are the most widespread geographically among poultry. G1-like strains (Guan et al. 1999; Peiris et al. 1999; Peiris 2009) have steadily migrated west from Southeast Asia to the Middle East (Davidson et al. 2014) and North and sub-Saharan Africa (El Houadfi et al. 2016). These viruses can be further subdivided based on relatedness and geographic distribution as “Eastern” (G1-h9.4.1) and “Western” (G1-h9.4.2) sublineages (Fig. 1; Dong et al. 2011; Fusaro et al. 2011; Nagy et al. 2017; Peacock et al. 2019). The G1-h9.4.1 strains are mainly found in South China, Vietnam, and Cambodia, and are mostly associated to minor poultry species such as quail, partridges, and guinea fowls. In contrast, G1-h9.4.1 isolates from west Asia and the Middle East have mostly been replaced by the most recent G1-h9.4.2 strains. The G1-h9.4.2 viruses are widely distributed and endemic in poultry in the Middle East (Israel, Jordan, Lebanon, Saudi Arabia, and the United Arab Emirates), Egypt, Bangladesh, Pakistan, regions of India, Afghanistan, and Nepal (Table 2; Tosh et al. 2008; Hosseini et al. 2017). G1-h9.4.2 viruses have been identified in poultry in Morocco, Ghana, Burkina Faso (Zecchin et al. 2017), and Uganda (Byarugaba et al. 2018). The G1-h9.4.2 viruses appear to have undergone reassortment at some point with cocirculating HPAI H7N3 and H5N1 viruses (Iqbal et al. 2009; Parvin et al. 2014). An isolate of a G1-h9.4.2-like virus was obtained in chickens in Germany, but it is not well established whether the original source of the virus were wild birds.

PUBLIC HEALTH RISKS OF H9N2 INFLUENZA A VIRUSES

Asian-origin H9N2 IAVs pose a threat to public health as zoonotic human infections are being reported in China, Hong Kong, Bangladesh, Egypt, Pakistan, and Oman (Table 2; Guan et al. 1999; Cameron et al. 2000; Butt et al. 2005; Banet-Noach et al. 2007; Jia et al. 2009; Fusaro et al. 2011; Blair et al. 2013; Monne et al. 2013; Yu et al. 2013; Shanmuganatham et al. 2014; Huang et al. 2015; Sun and Liu 2015; Wang et al. 2015b). As of June 2019, there have been 59 laboratory-confirmed H9 infections in humans with approximate equal distribution between sexes (public databases show sequence information for 29 of those cases) (Peacock et al. 2019). Young children, <8 years of age, were the most frequently affected. One subclinical infection was reported from an asymptomatic poultry worker in Pakistan (Ali et al. 2019). Serological studies among poultry workers in many enzootic countries such as India, Cambodia, China, Vietnam, Egypt, Hong Kong, Iran, Thailand, and Pakistan (Khan et al. 2015; Pusch and Suarez 2018) suggest significant exposure to H9 IAVs. Although serologic data are not always reliable, recent studies have tried to overcome these limitations through a number of approaches such as concurrent serotyping against multiple human and avian HA subtypes, meta-analysis, and longitudinal studies of poultry workers (Khan et al. 2015; Hoa et al. 2017). H9N2 IAV human isolates belong to either the G1-h9.4.1, G1-h9.4.2, or BJ94-h9.3 lineages, with HA sequences matching those circulating locally in poultry (Guo et al. 1999, 2000; Peiris et al. 1999; Huang et al. 2015; Sun and Liu 2015; Peacock et al. 2019). In most cases, contact with poultry was confirmed. However, in 11 cases there was no direct poultry exposure, which suggests indirect contact with H9N2 IAV or contact with non-poultry livestock. In addition, in 19 cases epidemiology data was not retrieved. No evidence of human-to-human transmission exists for H9 IAVs (Qi et al. 2013).

CLINICAL SIGNS, PATHOLOGY, AND INTERSPECIES TRANSMISSION

H9N2 infections in poultry are accompanied by substantial morbidity and increased susceptibility to secondary infections that could lead to high mortality (Nili and Asasi 2002; Brown et al. 2006; Kim et al. 2006; Banet-Noach et al. 2007; Khanna et al. 2008; Kwon et al. 2008; Lin et al. 2011a; Huang et al. 2012; Nili et al. 2013; Swayne et al. 2013; Jakhesara et al. 2014; Sieverding 2014; Śmietanka et al. 2014; Lee et al. 2016a; Zhu et al. 2018b). Clinical signs are associated with drops in water and food intake, reduced egg production, and diminished overall performances of the affected flock. Common respiratory signs include swelling of the sinuses and discharge from the eyes, nares, and mouth. In more severe cases, birds have severe dyspnea and difficulty breathing (Guo et al. 2000; Nili et al. 2013; Wang et al. 2015a, 2016a; Bonfante et al. 2018). H9N2 infections occur throughout the year, with lower morbidity in the summer (Xu et al. 2007b). At necropsy, affected birds typically show lesions of severe pulmonary consolidation with fibrinonecrotic exudate deposition, and mucus obstruction of bronchi and trachea (Nili et al. 2007; Sieverding 2014). Affected tracheas may present caseous casts formation in the lumen resulting in suffocation (Nili and Asasi 2002; Nili et al. 2007). H9N2 lesions are difficult to reproduce under experimental settings and confirmed only in a handful of cases (Guo et al. 2000; Wang et al. 2015a, 2016a; Bonfante et al. 2018). Turkeys are one of the most susceptible avian species showing considerable morbidity and mortality (Homme and Easterday 1970). Furthermore, high H9N2 virus isolation rates from apparently healthy chickens, ducks, and other minor poultry species (such as quail, partridges, chukar, pheasant, and guinea fowl) in live poultry markets have been reported (Xu et al. 2007b; Wang et al. 2014, 2015b). Interestingly, H9N2 viruses are frequently found in coinfections with H5N1 HPAI viruses in poultry flocks in Egypt in apparently healthy birds (Arafa et al. 2012; Monne et al. 2013; Naguib et al. 2017). Such observations further highlight the impact of H9N2 viruses as they are able to mask and promote transmission of HPAIVs in poultry. Cases of H9N2 virus replication in the infundibulum have been reported, which causes decrease in egg production and thinning of the egg shell with associated deformities (Wang et al. 2015a; Bonfante et al. 2018). In recent H9N2 outbreaks in chickens, virus replication was shown in various organs, including trachea, lung, spleen, and kidney, and especially nephrotropism was associated with increased mortality in chickens (Baron et al. 2013).

Molecular markers of IAV host switching from the natural reservoir—aquatic birds—to poultry are poorly defined (Naguib et al. 2015; Mosaad et al. 2018; Suttie et al. 2019). Most efforts are aimed at understanding interspecies transmission of IAVs from birds to mammals but not among different bird species (Imai et al. 2012). Numerous animal models have been used to study H9N2 IAV features under experimental settings. For avian species, chicken and quail are probably the most commonly used (Perez et al. 2003). Serial adaptation of a duck-origin H9N2 virus in quail leads to an expanded host range, with the virus acquiring the ability to replicate and cause disease in both chickens and mice (Hossain et al. 2008). In the same study, the adapted virus showed deletions in the stalk region of the NA, which is consistent with field observations of natural adaptation of influenza viruses in poultry (Hossain et al. 2008). A handful of pathogenesis studies have been also conducted in turkeys (Morales et al. 2009; Bonfante et al. 2013; Umar et al. 2015), chukars (Zhu et al. 2018b), ducks (Zhang et al. 2017a; Zhu et al. 2018b; Wang et al. 2019; Yang et al. 2019), and guinea fowl (Umar et al. 2016). In general, nonpoultry-adapted H9 IAVs showed limited replication and transmission in gallinaceous birds (Xu et al. 2012; Suarez and Puscha 2019). Overall, these studies highlight species-specific characteristics of H9N2 infections in avian species and confirmed the role of poultry in the perpetuation of H9 IAVs.

Surveillance in other domestic animal species, including pigs, minks, dogs, and cats have resulted in positive H9N2 virus isolations (Okamatsu et al. 2008; Shanmuganatham et al. 2014; Peacock et al. 2016; Xue et al. 2018). There is only a single report of an H9N2 virus of the BJ94 lineage isolated from a horse in Guanxi, China (He 2012). Like in other mammalian species, H9 infections in horses may be self-limiting sporadic events. Overall, pigs have the largest number of H9N2 isolations among mammalian species, in part explained by their proximity to poultry and also because of their known susceptibility to avian-origin IAVs. H9 IAVs do not seem to affect pigs, causing just mild respiratory signs. Most H9 isolates from pigs have been from farms in Hong Kong and China (Xu et al. 2004; Cong et al. 2007; Yu et al. 2011a; Wang et al. 2016b) and appear to be related to recurrent reintroductions rather than intraspecies circulation (Mancera Gracia et al. 2017b; Yang et al. 2017b). These observations are in line with experiments in pigs that show impaired transmission of H9N2 strains in pigs unless provided as reassortants in the background of swine-origin or human-origin IAV strains (Obadan et al. 2015; Wang et al. 2016b; Mancera Gracia et al. 2017a,b; Yang et al. 2017b).

Minks (in the family Mustelidae) are widely farmed for their fur and are highly susceptible to influenza viruses of both human and avian origins, including H9N2 (Åkerstedt et al. 2012; Yong-Feng et al. 2017; Xue et al. 2018). BJ94-like H9N2 viruses have been reported from minks in China, some of which carry the E627K mutation in PB2 associated with mammalian adaptation (Subbarao et al. 1993; Hatta et al. 2001; Peng et al. 2015). Serological studies in sea otters (also in the Mustelidae family) revealed antibodies to H9 viruses, perhaps resulting from exposure to H9 IAVs carried by sea birds (Capuano et al. 2017).

BJ94-lineage H9N2 IAVs have been isolated sporadically from dogs in China (Sun et al. 2013). Serological surveillance for H9N2 in stray dogs and feral cats showing flu-like clinical signs suggests infections from feeding on infected birds (Su et al. 2014; Zhou et al. 2015). Furthermore, recently emerged avian-origin H3N2 viruses isolated from dogs carry the PA segment derived from circulating H9N2 strains (Lee et al. 2016b) further highlighting the notion that H9N2 viruses can contribute to expanding the host range of other IAVs.

In the laboratory, mice, ferrets, guinea pigs, pigs, macaques, and tree shrews have been used to understand the pathogenesis and transmission of H9N2 viruses in mammals (Wan et al. 2008; Wu et al. 2009, 2010; Bi et al. 2011; Liu et al. 2011, 2014; Zhang et al. 2011; Li et al. 2012, 2018; Wang et al. 2012; Ku et al. 2014; Lin et al. 2014; Park et al. 2015; Sang et al. 2015b; Kamiki et al. 2018). These studies have allowed identification of a number of molecular markers associated with mammalian adaptation, transmission, and virulence (mostly in the mouse model). Details about these studies has been previously summarized (Perez and de Wit 2016). In this work, two updated tables summarize these findings: Table 3 contains general markers of avian and mammalian host tropism and airborne transmission found in H9 IAVs, and Table 4 contains virulence markers found in H9N2 strains but tested in the context of other IAV subtypes with zoonotic potential. In this context, it is important to note the role of the HA protein in interspecies transmission because it is commonly accepted that antigenic shift is the major driver in pandemic influenza. Noteworthy, the HA protein of most poultry-adapted H9N2 strains contain leucine at position 216 (L216, L226 in H3 HA numbering) within the receptor-binding site (RBS). L216 provides preferential binding to sialic acid (SA) linked to galactose by α2,6 linkage (SA α2,6Gal), which is typical of human-origin influenza viruses (Matrosovich et al. 2001; Wan and Perez 2007; Obadan et al. 2019). The second-most common amino acid signature at this position is glutamine Q216 (Q226 in H3 numbering) (Obadan et al. 2019). H9N2 strains with Q216 preferentially bind the α2,3 linkage SA receptors (SA α2,3Gal), more typical of avian-origin influenza viruses, but some show dual receptor specificity (Wan and Perez 2007; Obadan et al. 2019). The acquisition of HA L216 in H9N2 viruses is a sign of land-based poultry adaptation (Choi et al. 2004; Li et al. 2005; Xu et al. 2007a), consistent with the observation that both SA α2,3Gal and SA α2,6Gal receptors are present in the respiratory tract of some of these species (Gambaryan et al. 2002; Wan and Perez 2006; Guo et al. 2007; Kimble et al. 2010; Yu et al. 2011b). Other amino acids at position 216 are tolerated and have been found in nature, but do not seem favored in poultry (Obadan et al. 2019). Coincidentally, most Asian-origin poultry-adapted H9N2 strains can replicate in ferrets, although airborne transmission is typically inefficient (Wan et al. 2008; Sorrell et al. 2009; Kimble et al. 2011, 2014; Qiao et al. 2012; Scotch et al. 2014; SJCEIRS H9 Working Group

2013). However, airborne transmission of H9 subtype IAVs in ferrets is readily achieved if the H9 HA is tested in the background of human-adapted or swine-adapted strains (Table 3; Sorrell et al. 2009; Kimble et al. 2011, 2014). Two amino acid changes on the H9 HA, T179A (T189A in H3 numbering) in the HA1 region and R193G (R192G H3 numbering) in the HA2 region, have been shown to facilitate airborne transmission (Sorrell et al. 2009). This is consistent with the finding that the T179A or the A180V (189 and 190 H3 numbering, respectively) mutations provide enhanced human-like receptor binding (Srinivasan et al. 2013; Teng et al. 2016). Ferrets inoculated with field H9N2 isolates show mild clinical signs with the infection confined mostly to the upper respiratory tract (Wan et al. 2008). In contrast, ferrets inoculated with H9Nx avian/mammalian reassortants produce clinical signs and lung pathology associated with moderate to severe broncho-interstitial pneumonia, consistent with macrophages, lymphocyte and neutrophil infiltrations, alveolar edema, and rupture (Wan et al. 2008; Sorrell et al. 2009; Kimble et al. 2011, 2014; Gao et al. 2016). Recently, tree shrews have been proposed as an alternative to ferret and nonhuman primate models, being evolutionarily closer to humans. Tree shrews are similar to ferrets in terms of H9N2 IAV infection kinetics, pathogenesis, and SA receptors distribution (Li et al. 2018). Likewise, studies in Rhesus macaques inoculated with H9N2 revealed patterns of replication and pathogenesis similar to those seen in ferrets (Zhang et al. 2013). H9N2-inoculated macaques showed a biphasic peak in temperature at 1–2 and 5–6 days post-infection, displaying anorexia, lethargy, signs of dehydration, and cough. The lungs of these animals had multifocal to coalescing areas of dark reddish-tan consolidation associated with moderate to severe broncho-interstitial pneumonia histologically. Auxiliary findings were edema, hemorrhage, and fibrin deposition (Zhang et al. 2013; Nakayama et al. 2016).

SALIENT FEATURES OF THE H9 HA

In addition to the effects of amino acid changes within or near the RBS, the HA of H9 IAVs has some significant structural differences compared with other HA subtypes. The crystal structure of one prototypical poultry-adapted H9 HA protein was produced by Ha et al. (2001), which showed that the RBS displays the classical small hollow pocket on the globular head formed by a handful of nonconsecutive, highly conserved amino acids: Y91, P92, S130, W143, H173, and L184 (Y98, P99, S136, W153, H183, and L194, H3 numbering) (Table 5; Fig. 4). The globular head of the H9 HA lacks the lateral 130 loop that forms antigenic site A in the H3 and H5 HAs. This difference in the H9 HA results in two partially overlapping antigenic sites at the top of the molecule, sites I and II (Kaverin et al. 2004). Site I contains the amino acid residues 127, 129, 146–150, and 152 (equivalent to site A, 133, 135, 156–160, and 162, H3 numbering). Site II includes positions 135, 178, 179, 182, 183, 188, 189, and 216 (equivalent to site B, 145, 188, 189, 192, 193, 198, 199, and 226, H3 numbering) (Kaverin et al. 2004; Okamatsu et al. 2008; Peacock et al. 2018). Later work showed two other discrete, nonoverlapping antigenic sites, designated H9-A and H9-B to differentiate them from the previous H3 and H9 classification systems (Peacock et al. 2016, 2018). Site H9-A shares some similarities with the previously described site II and was shown to be moderately immunodominant compared with site H9-B. Few amino acid positions of antigenic relevance have been identified and tested (Table 5; Kaverin et al. 2004; Okamatsu et al. 2008; Burke and Smith 2014; Wan et al. 2014; Peacock et al. 2016; Adel et al. 2017). Many of these residues were identified in experiments using selection of escape mutants induced by murine mAbs (Kaverin et al. 2004; Peacock et al. 2016, 2017), but the extent of the type of amino acid changes that modulate virus escape in the field remain largely undefined.

Glycosylation of HA (and NA) influences immune recognition and receptor binding (and enzyme function). In general, avian-origin IAVs contain fewer potential glycosylation sites on the HA than human IAVs (Tate et al. 2014). Changes in glycosylation sites on the H9 HA have been reported both under experimental settings and following natural evolution of IAVs in the field (Kandeil et al. 2014; Peacock et al. 2016, 2018). H9 escape mutants generated with both mouse monoclonal and chicken polyclonal antibodies give rise to glycosylation sites at positions T127N, K131N, A150T, L150S, T188N, and D189N (T133N, A160T, L160S, T198N, and D199N, H3 numbering) (Fig. 4; Peacock et al. 2018). Five glycosylation sites, 11, 90, 127, 281, and 288 (21, 97, 133, 290, and 297, H3 numbering) were found commonly in all HAs of H9N2 Egyptian isolates from 2011 and 2013 (Kandeil et al. 2014). Other glycosylation sites such as 188 and 200 (198 and 210, H3 numbering) were lost from all Egyptian isolates when compared with G1-like viruses (Kandeil et al. 2014).

The HA of H9 viruses can be cleaved by extracellular proteases of the respiratory tract-like human transmembrane protease, serine S1 member 2 (TMPRSS2) and human airway trypsin-like protease (HAT) likewise to H7N9 viruses (Baron et al. 2013; Sakai et al. 2014; Tarnow et al. 2014). Interestingly, H9 HAs with the cleavage site sequence RSSR/GL or RSRR/GL can be cleaved by matriptase, a protease widely expressed in most epithelia. Matriptase is abundantly expressed in the kidney and may explain in part the nephrotropism of some H9N2 viruses (Baron et al. 2013). After loss of a glycosylation site, cleavage by furin has been observed, which is atypical in LPAIVs, but may influence the ability of H9N2 viruses to spread in poultry (Tse et al. 2014). Also, a single amino acid substitution N148D (158, H3 numbering) is important for the attenuation of H9N2 virus disease in mice and chickens (Jin et al. 2019; Suttie et al. 2019).

VACCINATION, PREVENTION, AND CONTROL

The economic impact of H9N2 infection in poultry have led many countries to adopt vaccination programs as means for prevention and control. China implemented a long-term vaccination program in chicken farms as early as 1998 (Li et al. 2005; Zhang et al. 2008). More recently, Italy, Israel, South Korea, Morocco, Pakistan, Egypt, Iran, and the United Arab Emirates have adopted similar vaccination efforts. Vaccination, typically in the form of an inactivated virus, has been proven effective in mitigating clinical disease and production losses, but has failed to control H9N2 virus spread. Evidence of antigenic drift driven by poor vaccine antigenic match has been observed (Zhang et al. 2008; Iqbal et al. 2009; Park et al. 2011; Jiang et al. 2012; Shanmuganatham et al. 2013, 2014; Lee et al. 2016a; Marinova-Petkova et al. 2016; Ali et al. 2018). As for other influenza vaccines, matching the seed vaccine strain with the viruses circulating in the field is the key to successful immunization efforts (Suarez and Puscha 2019). Overall, there is an increasing need to develop alternative vaccination strategies and systems that can better contain the spread of

H9N2 IAVs in poultry (Song et al. 2007; Cai et al. 2011; Lin et al. 2011b; Ducatez et al. 2016; Hajam et al. 2018; Liu et al. 2018, 2019; Xu et al. 2019).

H9-LIKE VIRUSES IN FRUIT BATS

Fruit bats are a major reservoir of emerging viral pathogens. Surveillance studies in two different species of fruit bats, the little yellow-shouldered bat *(Sturnira lilium)* in Guatemala and the flat-faced fruit-eating bat *(Artibeus jamaicensis)* in Peru, led to the characterization of type A–like influenza viruses, H17N10 and H18N11, respectively (Tong et al. 2012, 2013). More recently, surveillance studies in Egypt revealed the presence of another bat virus, more similar to avian-origin influenza virus strains than those previously characterized in Central and South America (Tong et al. 2012; Campos et al. 2019; Kandeil et al. 2019). The Egyptian fruit bat *(Rousettus aegyptiacus)* virus isolates contain an HA segment with common ancestry with other H9 viruses, and low-level cross-reactivity with serum raised against H9N2 viruses (Kandeil et al. 2019). Bats were seropositive for the isolated viruses and consistent with sero-surveillance studies in Ghana that showed that 30% of frugivorous bat sera contained antibodies that recognized H9 IAVs and, to a lesser extent, H8 and H12 IAVs (Freidl et al. 2015). Unlike the H17N10 and H18N11 viruses, the H9N2-like bat virus was able to grow in eggs and MDCK cells and displayed an avian-like SA-binding specificity. In addition, the bat H9-like viruses contain markers of mammalian adaptation (S199 and D701N in PB2; 13P in PB1; N55, Y241, and S404 in PA; K357 and E455 in NP; N20 in M2) and are able to infect mice (Kandeil et al. 2019).

CONCLUDING REMARKS

In recent years, H9N2 IAVs established stable poultry adapted lineages whose prevalence have increased exponentially, affecting many countries worldwide. Limited surveillance, subclinical circulation in poultry, less than optimal vaccination approaches, and the nonreportable status of H9 IAVs have contributed to their perpetuation in poultry and the emergence of zoonotic strains of pandemic concern. H9N2 have been isolated from numerous sylvatic and domestic animals, such as wild birds, pigs, horses, dogs, cats, pikas, and minks, which highlight their potential for expanded host range. Some H9s IAVs naturally possess internal gene cassettes (e.g., genotype 57), pH stable HA proteins, and receptor-binding specificities, which facilitate infection and replication in human host cells (Thuy et al. 2016; Zhu et al. 2018a; Han et al. 2019; Wang et al. 2019). Several novel emerging zoonotic IAVs such as H7N9, H5N1, H10N8, and H5N6 obtained their internal gene cassette from previous reassortment events with H9N2 viruses. It is safe to speculate that H9s IAVs from poultry have adopted, not only on the HA gene segment but also in the rest of the genome, molecular features that make them more similar to human influenza strains than possibly any other IAV circulating in the avian reservoir.

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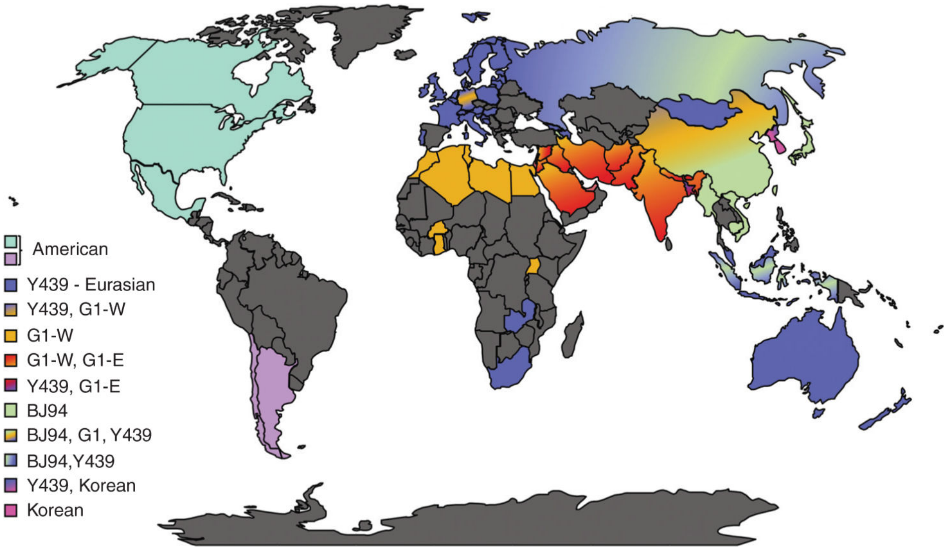

Figure 1.

World map and approximate lineage/clade circulation in which H9 subtype influenza A viruses (IAVs) have been reported. Please see main text for full description of lineages/ clades. The H9N2 subtype combination is the most commonly found in nature. Dark gray corresponds to areas of the world where the presence or circulation of H9 IAVs is unknown.

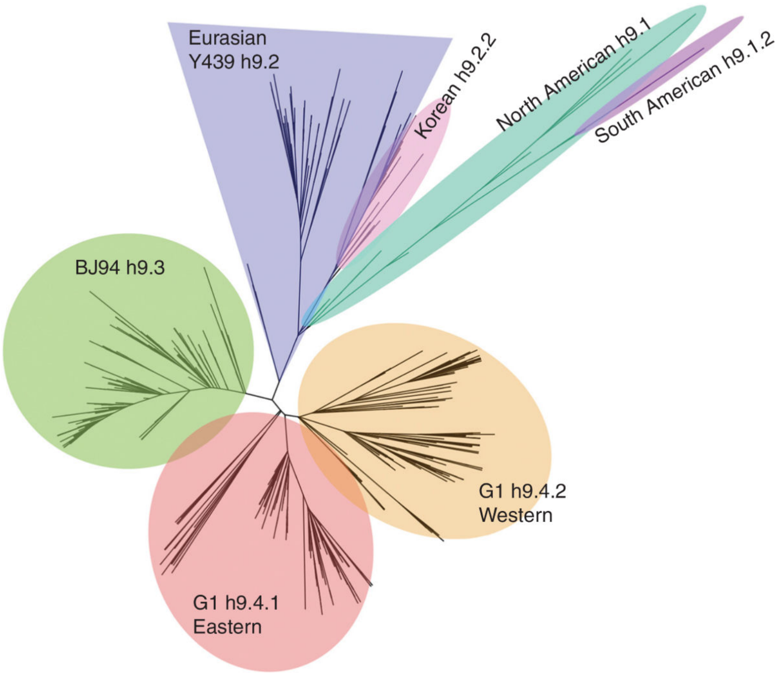

Figure 2.

Global phylogenetic star tree (BEAUTi and BEAST v1.10.4) of H9 influenza A viruses (IAVs) based on the HA1 nucleotide sequence. H9 major lineages are color coded, respectively: h9.1—North American, teal; h9.1.2—South American, purple; h9.2—Y439, blue; h9.2.2—Korean, pink; h9.3—BJ94, green; h9.4.1—G1-Eastern, red; h9.4.2—G1-Western, orange.

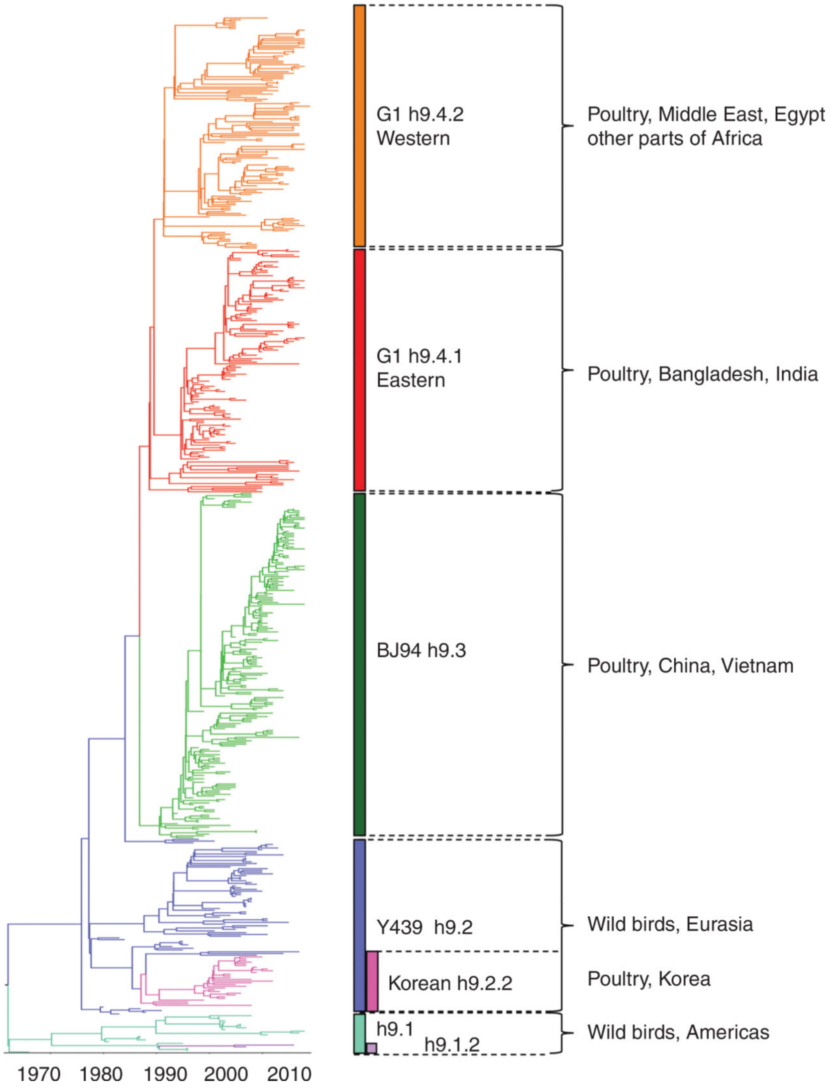

Figure 3.

Temporal most recent common ancestor (TMRCA) phylogenetic analysis generated with BEAUTi and BEAST v1.10.4 under a strict molecular clock of 600 H9N2 influenza A viruses (IAVs) HA1 nucleotide sequences aligned in MUSCLE 3.8.425 (IRD and GISAID databases at August 2019). H9N2 clades are color coded, respectively: h9.1—North American, teal; h9.1.2—South American, purple; h9.2—Y439, blue; h9.2.2—Korean, pink; h9.3—BJ94, green; h9.4.1—G1-Eastern, red; h9.4.2—G1-Western, orange. Animal reservoir and countries with most common isolations for each lineage are shown.

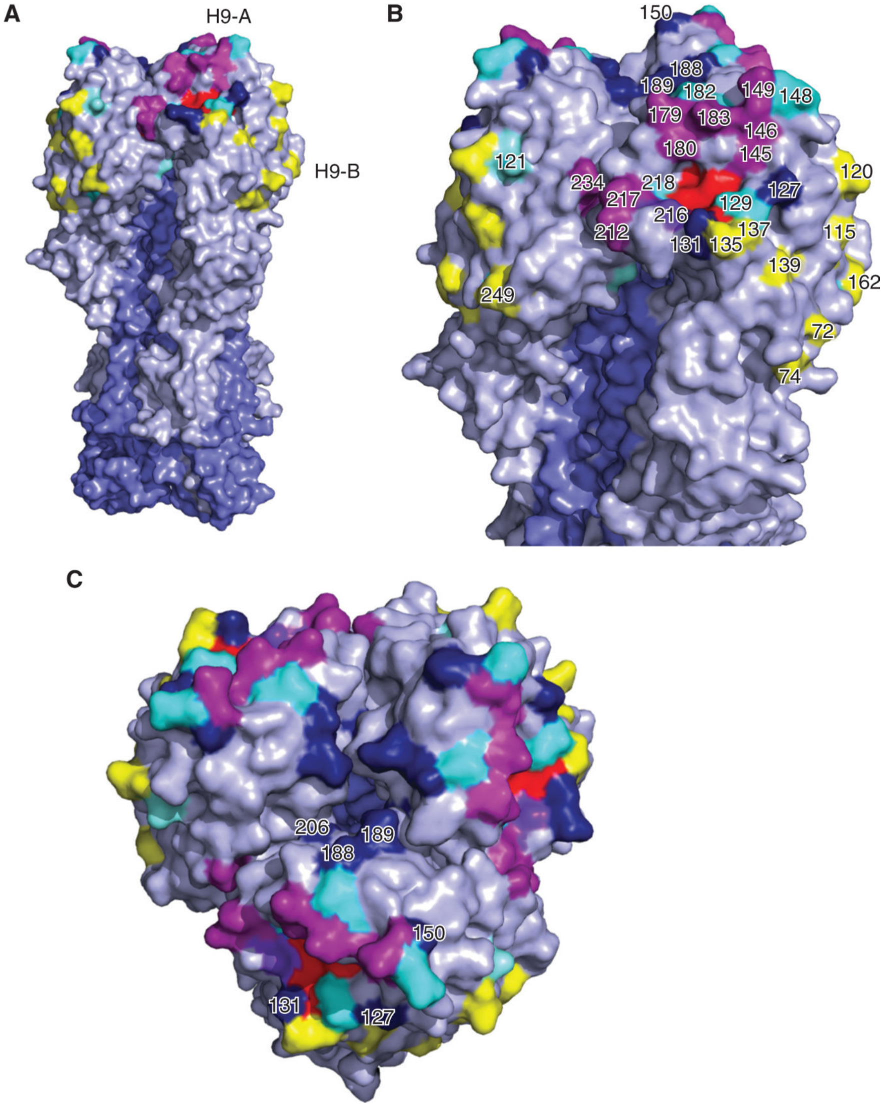

Figure 4.

Relevant structural features of the hemagglutinin (HA) of the H9 subtype. Homotrimers of the HA crystal structure of A/swine/Hong Kong/9/1998 (Protein databank ID:1JSD) colored in PyMOL. Selected receptor-binding site (RBS) residues are colored in red. HA1 and HA2 portions are highlighted in gray and slate blue, respectively. (*A*) The full HA homotrimer is shown. (*B*,*C*) Details of the HA globular head. Shown are the antigenic site H9-A (magenta) and H9-B (yellow). Other antigenic residues without assigned site classification are shown in cyan. Potential glycosylation sites are colored in dark blue.

Table 1.

H9Nx frequencies in different animal species as of September 2019

		Avian												Mammalian						
	No.	**Chicken**	**Turkey**	**Quail**	**Partridge**	**Peafowl**	**Pheasant**	**Waterfowl**	**Shorebird**	**Pigeon**	**Ostrich**	**Bustard**	**Other**	**Swine**	**Horse**	**Mink**	**Pika**	**Dog**	**Bat**	**Human**
H9Nx	2178	1886	0	8	0	0	0	208	14	58	0	0	0	0	0	0	0	0	0	0
H9N1	33	1	0	0	0	1	0	13	17	0	0	0	0	0	0	0	0	0	0	0
H9N2	7250	5850	108	150	35	17	37	616	53	202	10	7	70	55	1	13	1	1	1	23
H9N3	10	0	0	0	0	0	0	4	5	0	0	0	1	0	0	0	0	0	0	0
H9N4	3	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0
H9N5	22	0	0	0	0	0	0	1	21	0	0	0	0	0	0	0	0	0	0	0
H9N6	7	0	0	0	0	0	0	4	3	0	0	0	0	0	0	0	0	0	0	0
H9N7	10	0	1	0	0	0	0	1	8	0	0	0	0	0	0	0	0	0	0	0
H9N8	6	1	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0
H9N9	16	2	1	0	0	0	0	0	13	0	0	0	0	0	0	0	0	0	0	0
Total	9535	7740	110	158	35	18	37	852	137	260	10	7	71	55	1	13	1	1	1	23

Sequence data deposited in the Influenza Research Database (fludb.org).

The darker the color green, the higher the number of cases. Transition to orange indicates the greatest number for each H9N? combination.

Table 2.

Countries with reported H9 subtype IAV isolations and corresponding lineages

H9N2 lineage	Country	Host	Confirmed human cases[a]
North American (h9.1.1)	Canada	Wild waterfowl (mallard)	
	Mexico	American wigeon	
	United States	Chicken, quail, turkey, shorebird, waterfowl	
	China	Chicken, plateau pikas, wild waterfowl	
South American (h9.1.2)	Argentina	Rosy-billed pochard	
	Chile	Mallard duck	
Y439 (h9.2)	Bangladesh	Chicken, quail, wild waterfowl	
	Belgium	Chicken, duck	
	Cambodia	Chicken	
	France	Turkey, waterfowl	
	Germany	Chicken, turkey	
	Hungary	Chicken	
	Indonesia	Chicken	
	Italy	Chicken, turkey	
	Malaysia	Waterfowl	
	Netherland	Chicken, turkey	
	Poland	Turkey, duck	
	South Africa	Ostrich	
	North Korea	Chicken	
	South Korea	Chicken, guinea fowl, duck, goose, pheasant, wild waterfowl, dove	
	United Kingdom	Chicken, turkey	
	Vietnam	Chicken, quail	
Korean (h9.2.2)	North Korea	Chicken	
	South Korea	Chicken, guinea fowl, duck, goose, pheasant, wild waterfowl, dove	
BJ94 (h9.3)	Cambodia	Chicken	
	China	Chicken, quail, turkey, goose, duck, partridge, pheasant, guinea fowl, waterfowl, shorebirds	1998 (5), 1999 (1), 2013 (2), 2014 (2), 2015 (5), 2016 (2), 2017 (4), 2018 (2)
	Hong Kong	Chicken, guinea fowl, duck, goose, pheasant, wild waterfowl, partridges	2003 (1)
	Indonesia	Chicken	

H9N2 lineage	Country	Host	Confirmed human cases[a]
	Japan	Chicken, imported meat	
	Myanmar	Chicken	
	Russia	Teal	
	Vietnam	Chicken, quail	
G1-Eastern (h9.4.1)	Cambodia	Chicken	
	China	Chicken, quail, turkey, goose, duck, partridge, pheasant, guinea fowl, waterfowl, shorebirds	2004 (1)
	Hong Kong	Chicken, guinea fowl, duck, goose, pheasant, wild waterfowl, partridges	1999 (2), 2009 (2)
	Vietnam	Chicken, quail	
G1-Western (h9.4.2)	Afghanistan	Chicken	
	Algeria	Chicken	
	Bangladesh	Chicken, quail	2011 (1)
	Burkina Faso	Chicken	
	Egypt	Chicken, quail, turkey	
	Germany	Chicken	
	Ghana	Chicken	
	India	Chicken	
	Iran	Chicken	
	Iraq	Chicken	
	Israel	Chicken, turkey, ostrich	
	Jordan	Chicken	
	Kuwait	Chicken	
	Lebanon	Chicken, quail	
	Libya	Chicken	
	Morocco	Chicken	
	Nepal	Chicken	
	Oman	Chicken	2019 (1)
	Pakistan	Chicken, partridges	2015 (1)
	Russia	Chicken	
	Saudi Arabia	Chicken	
	Tunisia	Chicken, turkey	
	United Arab Emirates	Chicken, quail	

H9N2 lineage	Country	Host	Confirmed human cases[a]
Unknown	Uganda	Chicken	
	Colombia	Chicken	
	Laos	Chicken	
	Nigeria	Chicken	
	Romania	n/a	
	Thailand	n/a	

[a] Year of isolation is followed by the number of laboratory-confirmed human cases in parentheses.

Table 3.

Amino acid residues associated with increased tropism of H9 influenza A viruses (IAVs) in avian or mammalian hosts

Gene	Protein	Residue	Tropism		Host[a]	Reference(s)
			Avian	Mammalian		
1	PB2	58	T	I	Ferret	Kimble et al. 2011
		64	M	T	Chicken, mice	Kandeil et al. 2014; Naguib et al. 2015; Mosaad et al. 2018
		147	M	L	Mice	Wang et al. 2012
		195	D	N	Guinea pigs, mammalian cell lines	Sang et al. 2015a; Suttie et al. 2019
		199	A	S	Chickens, bat	Kandeil et al. 2014, 2019; Mosaad et al. 2018
		250	V	G	Mice	Wang et al. 2012
		253	D	N	Enhanced ferret, mammalian cell lines	Mok et al. 2011; Kimble et al. 2014
		271	T	A	Chicken, human cells, mice	Hayashi et al. 2015; Mosaad et al. 2018; Long et al. 2019
		292	I	V	Chicken, mice	Naguib et al. 2015; Suttie et al. 2019
		318	K	R	Chicken, mice, mammalian cell lines	Kandeil et al. 2014; Naguib et al. 2015
		374	L	I	Ferret	Sorrell et al. 2009
		404	F	L	Mice	Liu et al. 2014, 2015
		535	M	L	Mammalian and avian cells, mice	Lloren et al. 2017
		588	A	I	Chicken, mammalian, human and avian cells, mice	Xiao et al. 2016; Lloren et al. 2017; Mosaad et al. 2018; Suttie et al. 2019
		591	Q	K	Mice, mammalian cell lines	Mok et al. 2011; Long et al. 2019; Suttie et al. 2019
		627	V/E	K	Chicken, mice, human, ferret, mammalian cell lines	Hatta et al. 2001; Le et al. 2009; Wang et al. 2012; Kandeil et al. 2014; Li et al. 2014; Mosaad et al. 2018
		661	A	T	Chicken, mice, bat	Kandeil et al. 2014; Naguib et al. 2015
		701	D	N	Ferrets, mice, bat, mammalian cell lines	Li et al. 2014; Naguib et al. 2015; Kandeil et al. 2019
		714	S/G	R	Mammalian cell lines, mice	Sediri et al. 2016; Suttie et al. 2019
		740	D	N	Mice	Park et al. 2015
2	PB1	13	L	P	Chicken, mice, mammalian cell lines	Gabriel et al. 2005; Kandeil et al. 2014, 2019; Naguib et al. 2015
		120	D	N	Ferret	Sorrell et al. 2009; Kimble et al. 2011, 2014
		261	S	N	Ferret	Sorrell et al. 2009; Kimble et al. 2011, 2014
		577	K	E	Mice	Kamiki et al. 2018; Suttie et al. 2019
	PB1-F2	73	K	R	Chicken, mice	Naguib et al. 2015; Mosaad et al. 2018
		82	L	S	Chicken, mice	Perales et al. 2000; Naguib et al. 2015; Mosaad et al. 2018

Gene	Protein	Residue	Tropism		Host[a]	Reference(s)
			Avian	Mammalian		
3	PA	PA-X	-	-	Mice	Gao et al. 2015a,b
		3	D	V	Mice	Liu et al. 2014
		26	K	E	Ferret	Kimble et al. 2014
		28	P	L	Chicken, bat	Mosaad et al. 2018; Kandeil et al. 2019
		55	D	N	Chicken, mice, bat	Kandeil et al. 2014, 2019; Mosaad et al. 2018
		100	V	A	Chicken, mice	Kandeil et al. 2014; Naguib et al. 2015; Mosaad et al. 2018
		160	V	D	Mice	Park et al. 2015
		225	S/G	C	Chicken, mice	Kandeil et al. 2014; Liu et al. 2014; Mosaad et al. 2018
			S	R	Mice	
		241	C	Y	Bat, chicken	Kandeil et al. 2014, 2019
		356	K	R	Chicken, mice, human	Xu et al. 2016; Mosaad et al. 2018; Suttie et al. 2019
		404	A/N	S	Chicken, bat, human, mice	Kandeil et al. 2014, 2019; Naguib et al. 2015
		549	L	I	Mice	Park et al. 2015
		615	K	N	Chicken, mice	Kandeil et al. 2014; Naguib et al. 2015; Mosaad et al. 2018
4	HA[b]	71	L	F	Mice	Liu et al. 2014
		104	A	V	Ferret	Kimble et al. 2011
		148	N	D	Mice	Jin et al. 2019; Suttie et al. 2019
		179	T	A	Enhanced replication in ferrets, transmitted via aerosols among ferrets	Sorrell et al. 2009
		180	E	D	Ferret, mice	Sorrell et al. 2009; Yang et al. 2017a,b
			T	V	Fluman	Teng et al. 2016; Suttie et al. 2019
		182	R	G	Enhanced replication in ferrets, aerosols transmission among ferrets	Sorrell et al. 2009
		183	K	S	Swine, mice; increased virus binding to α2–6, enhanced replication in mammalian cells	Yang et al. 2017a,b
			N	D	Mice	
		190	T	A	Swine; affinity to α2,6	Peiris et al. 1999; Lloren et al. 2017
		202	T	I	Swine cell lines, mice	Yang et al. 2017a,b
		216	Q/L	Q/L	L216 increased virus binding to α2–6, enhanced replication in mammalian cells and ferrets, enhanced contact transmission in ferrets	Wan and Perez 2007; Wang et al. 2012

Gene	Protein	Residue	Tropism Avian	Tropism Mammalian	Host[a]	Reference(s)
		217	Q	P	Guinea pigs, mammalian cell lines	Sang et al. 2015a; Suttie et al. 2019
	HA2	192	R	G	Enhanced airborne transmission in ferrets	Sorrell et al. 2009
5	NP	105	V	M	Mice	Liu et al. 2014
		109	I	V	Chicken, mice	Kandeil et al. 2014; Naguib et al. 2015
		214	R	K	Chicken, mice	Kandeil et al. 2014; Naguib et al. 2015
		357		K	Chicken, mice, bat	Kandeil et al. 2014, 2019
		398	K	Q	Chicken, mice	Kandeil et al. 2014; Naguib et al. 2015
		434	E	K	Guinea pigs, mammalian cell lines	Sang et al. 2015a; Suttie et al. 2019
		455		E	Chicken, bat	Kandeil et al. 2014, 2019
6	NA	27	A	T	Mice	Liu et al. 2014
		30	A	T	Ferret	Kimble et al. 2011
		62–64		Δ	Mice	Guo et al. 2000; Liu et al. 2014
		69–73		Δ	Human	Yu et al. 2016
7	M1	15	V	I	Bat, chicken, mammalian cell lines	Kandeil et al. 2014, 2019
		110	H	Y	Ferret	Sorrell et al. 2009
		166	A	V	Mice	Liu et al. 2014
		210	R	K	Mice	Wang et al. 2012
	M2	20	S	N	Chicken, quail, bat	Kandeil et al. 2014, 2019
8	NS1	227	E	K/R	Chicken, mice	Kandeil et al. 2014; Naguib et al. 2015

[a] Hosts in which the mammalian marker was identified and/or tested.

[b] HA residue positions adjusted to mature H9 HA numbering. All the other genes follow original numbering as stated in the reference.

Table 4.

Virulence determinants identified in H9 influenza A viruses (IAVs)

Gene	Protein	Residue	Virulence		Effect	Reference(s)
			Lower	Higher		
1	PB2	147	M	L	Increased virulence in mice	Wang et al. 2012; Naguib et al. 2015; Mosaad et al. 2018
		250	V	G	Increase replication, transmission and pathogenicity in chickens	Naguib et al. 2015; Mosaad et al. 2018
		253	D	N	Increase replication and transmission in ferrets	Yamada et al. 2010; Mok et al. 2011; Kimble et al. 2014
		292	I	V	Increased virulence in mice	Gao et al. 2019; Suttie et al. 2019
		404	F	L	Increased virulence in mice	Liu et al. 2014
		504	I	V	Increased virulence in mice	Kandeil et al. 2014, 2019; Naguib et al. 2015
		588	T	I	Increased virulence in mice	Xiao et al. 2016; Suttie et al. 2019
			A	V	Higher polymerase activity; efficient replication in mammalian and avian cells; increased virulence in mice	Xiao et al. 2016; Lloren et al. 2017
		591	Q	K/R	Increased virulence in mice	Mok et al. 2011; Suttie et al. 2019
		627	E	V/K	Increased virulence in mice	Wang et al. 2012; Li et al. 2014; Naguib et al. 2015; Suttie et al. 2019
		701	D	N	Increase replication and transmission in ferrets; increase virulence in mice and chickens	Yamada et al. 2010; Kandeil et al. 2014; Kimble et al. 2014; Naguib et al. 2015
		714	S	R	Increased virulence in mice	Sediri et al. 2016; Suttie et al. 2019
2	PB1	317	M/V	I	Increase replication, transmission and pathogenicity in chickens	Naguib et al. 2015
		577	K	E	Increased virulence in mice	Kamiki et al. 2018; Suttie et al. 2019
	PB1-F2	66	N	S	Increased virulence in mice	Conenello et al. 2011; Naguib et al. 2015
3	PA	127	I	V	Increase replication, transmission and pathogenicity in chickens	Naguib et al. 2015
		356	K	R	Increased virulence in mice	Xu et al. 2016; Suttie et al. 2019
		550	I	L	Increase replication, transmission and pathogenicity in chickens	Naguib et al. 2015
		672	F	L	Increase replication, transmission and pathogenicity in chickens	Zhong et al. 2014; Naguib et al. 2015; Mosaad et al. 2018
		PA-X	Δ	PA-X	Virulence in mice	Gao et al. 2015a,b; Suttie et al. 2019
4	HA	148	D	N	Increased virulence in chickens and mice	Jin et al. 2019; Suttie et al. 2019
		166	N	S	Increased virulence in mice	Park et al. 2015
		188	N	T	Increase replication and transmission in ferrets	Kaverin et al. 2004
6	NA	45–46		Δ	Increased virulence in chickens	Guo et al. 2000; Liu et al. 2014
		62–64		Δ	Increased virulence in chickens	Guo et al. 2000; Lin et al. 2014; Liu et al. 2014
		119	E	D	Reduced susceptibility to zanamivir	Kode et al. 2019; Suttie et al. 2019

Gene	Protein	Residue	Virulence		Effect	Reference(s)
			Lower	Higher		
7	M2	31	S	N/G	Increased resistance to amantadine and rimantadine	Ilyushina et al. 2005; Suttie et al. 2019
		64	P	S/A/F	Increase replication, transmission and pathogenicity in chickens	Naguib et al. 2015
		69	L	P	Increase replication, transmission and pathogenicity in chickens	Naguib et al. 2015
8	NS1	230–237	Elongation		Increased replication and inflammatory cytokine production in chickens	Kong et al. 2015; Suttie et al. 2019
		2	D	N	Increase replication and transmission in ferrets	Kimble et al. 2014
		42	A/P	S	Increase replication, transmission and pathogenicity in chickens	Naguib et al. 2015; Yu et al. 2016
		103	F	L	Increase replication, transmission and pathogenicity in chickens	Naguib et al. 2015
		106	I	M	Increase virulence in mice and chickens	Ayllon et al. 2014; Kandeil et al. 2014; Naguib et al. 2015
		189	D/G	N	Increase replication, transmission and pathogenicity in chickens	Naguib et al. 2015
	NS2/NEP	31	M	I	Increase replication, transmission and pathogenicity in chickens	Naguib et al. 2015
		56	H/L	Y	Increase replication, transmission and pathogenicity in chickens	Naguib et al. 2015

All HA positions follow the H9 numbering. All the other genes follow original numbering as stated in the references. Not necessarily tested in H9 IAV backgrounds.

Table 5.

Reported residues of antigenic relevance identified with mouse monoclonal and chicken polyclonal antibodies

Residue[a]	Conservation %[b]	Antigenic site	Most frequent amino acid residue in nature[c]						Published mAb escape	Impact on chicken antisera	Reference(s)
			American	Y439	Korean	G1-E	G1-W	BJ94			
72	82.2	H9-B	G	G	G	G/E	G	G/E	G-E	G-E	Okamatsu et al. 2008; Peacock et al. 2018
74	73.9		K	K	K	R	R	R	R-K, R-G[d]	R-G	Zhu et al. 2015; Peacock et al. 2018
98	98.9		L	L	L	L	L	L	L-Q	–	Okamatsu et al. 2008; Peacock et al. 2018
109	56.4		K/S/R	N	R	S	S	R/S	S-R, S-I	–	Kaverin et al. 2004; Peacock et al. 2018
115	91.5	H9-B	Q	Q	L	Q	Q	Q/L	Q-P, Q-R, Q-L	Q-P	Peacock et al. 2016, 2018; Jin et al. 2019
120	81.7	H9-B	T/R	T	R	T/A	T/S	T/S	T-K	–	Peacock et al. 2016, 2018
121	54.6		I/T	I	T	I	I	I	I-T[d]	I-T	Peacock et al. 2018
127	49	Overlap	S	S	T	T/D	T/S	S/D	S-N[e]	T-N[e]	Kaverin et al. 2004; Okamatsu et al. 2008; Peacock et al. 2018
129	99.6	Site I	T	T	T	T	T	T	T-A, T-K	T-K	Kaverin et al. 2004; Peacock et al. 2018
131	84.8		K/R	K	K	K	K	K/T	K-N[e], K-I[d], K-R[d], A-R[d]	K-I	Okamatsu et al. 2008; Peacock et al. 2018
135	62.4	H9-B/site II	N/D	N	G/N/D	D/G	D	D/G	D-E, D-N, D-G[d]	D-G	Kaverin et al. 2004; Wan et al. 2014; Peacock et al. 2018
137	99.8		F	F	F	F	F	F	F-L	F-L	Kaverin et al. 2004; Peacock et al. 2018
139	98.9	H9-B	R	R	R	R	R	R	R-G, R-M	–	Peacock et al. 2016, 2018
145	99.8	H9-A	T	T	T	T	T	T	T-I	T-I, S-N	Ping et al. 2008; Zhu et al. 2015; Peacock et al. 2016, 2018
146	70.8	H9-A/site I	H	H	H	Q	Q	Q/R	Q-K	Q-H	Wan et al. 2014; Peacock et al. 2018
147	99.3	Site I	K	K	K	K	K	K	K-T	–	Kaverin et al. 2004; Wan et al. 2014; Peacock et al. 2018
148	59.9	Site I	S	S	S	S	N	N/S/D	N-D	N-D	Okamatsu et al. 2008; Zhu et al. 2015; Peacock et al. 2018
149	54.3	H9-A/site I	N	N	N	G	G	N	N-D, N-K	G-D/K	Wan et al. 2014; Peacock et al. 2018

			Most frequent amino acid residue in nature[c]								
Residue[a]	Conservation %[b]	Antigenic site	American	Y439	Korean	G1-E	G1-W	BJ94	Published mAb escape	Impact on chicken antisera	Reference(s)
150	33	Site I	S	S	S	S/N/F	L/N/F/V/G	A/N/D/E	A-T[e], A-D A/N-V/L[d], A-F[d], F-L[d], F-S- T[d]	A-L/F, L-S[e]/A	Wan et al. 2014; Peacock et al. 2018
152	99.5	Site I	P	P	P	P	P	P	P-L	–	Kaverin et al. 2004; Peacock et al. 2018
162	50.6	H9-B	E/K	E	W	R	R/Q	Q/R	R-W	–	Peacock et al. 2016, 2018
178	95.5	Site II	D	D	D	D/E	D	D/E	D-V	–	Wan et al. 2014; Peacock et al. 2018
179	93.6	H9-A/overlap	T	T	T	T	T	T/D	T-A	T-N	Kaverin et al. 2004; Zhu et al. 2015; Peacock et al. 2018
180	46.8	H9-A	E	E	E	A/E	A/T/V	A/V/T	A-E-T[d], A-V[d], E-D[d]	A-E	Peacock et al. 2018
182	85.7		N/T	T	M	T	T	T/R	T-I	T-R	Okamatsu et al. 2008; Peacock et al. 2018
183	86.8	H9-A/site II	N/D	N	N	N	N	N/D	N-D, N-S, N-T, N-E/T[d]	N-T	Kaverin et al. 2004; Okamatsu et al. 2008; Wan et al. 2014; Peacock et al. 2016, 2018
188	75	Overlap	T/A	A/D	A	T/N	T/N	T	T-N[e]	T-N[e]	Kaverin et al. 2004; Peacock et al. 2018
189	98.7		D	D	D	D	D	D	D-N[e]	D-N[e]	Wan et al. 2014; Peacock et al. 2018; Jin et al. 2019
195	59.7		T	T	T	T/A	T	A/T	A-T[d]	NT	Peacock et al. 2018
198	46.6		D	D	D	D/N	N/D	E/D	D-N	–	Peacock et al. 2018
200	68.6		N	N	N	N	D	N/D	N-D	N-D	Wan et al. 2014; Jin et al. 2019
212	96.9	H9-A	L	L	L/I	L	L	L	L-H, L-P	–	Okamatsu et al. 2008; Peacock et al. 2016, 2018
216	62.3	Site II	Q	Q	Q	Q/L	L/Q	L/Q	L-Q	L-Q	Kaverin et al. 2004; Peacock et al. 2018
217	55.4	H9-A	Q	Q	Q	Q	I	Q/M	I-T	I-L/M/Q	Peacock et al. 2016, 2018
234	99.8	H9-A	R	R	R	R	R	R	R-Q, L-M	–	Peacock et al. 2016, 2018; Jin et al. 2019
249	65.7		V	I	I	I/V	V/I	I	I-V/A[d]	I-V	Peacock et al. 2018
264	51.4		N	N	N/K	N/K	N/K/S	K/N	K-N[d]	NT	Peacock et al. 2018

Residue[a]	Conservation %[b]	Antigenic site	Most frequent amino acid residue in nature[c]						Published mAb escape	Impact on chicken antisera	Reference(s)
			American	Y439	Korean	G1-E	G1-W	BJ94			
276	83.7		R/K	K	K	K	K	K/R	K-R[d]	NT	Peacock et al. 2018
288	61.9		I/V	V	V	I	I/V	V	I-V[d]	NT	Peacock et al. 2018
306	99.3		K	K	K	K	K	K	I-K[d]	NT	Peacock et al. 2018

The most frequent residues at those positions for each H9N2 lineage are reported.

(NT) Not tested.

[a] All HA positions follow the H9 numbering. Percent of conservation at selected residues is based on the global avian H9N2 HA1 amino acid alignment of 600 sequences from the analysis in Figure 3 with Scop3D entropy calculation (Vermeire et al. 2015).

[b] Percent of conservation.

[c] Most frequent amino acid residue at selected positions based on global H9 phylogenetic analysis of HA1.

[d] Identified by integrated modeling of HI titers and gene sequence data.

[e] Potential glycosylation sites.