



# Mammalian pathogenicity and transmissibility of a reassortant Eurasian avian-like A(H1N1v) influenza virus associated with human infection in China (2015)

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## ABSTRACT

Swine-origin (variant) H1 influenza A viruses associated with numerous human infections in North America in recent years have been extensively studied *in vitro* and in mammalian models to determine their pandemic potential. However, limited information is available on Eurasian avian-like lineage variant H1 influenza viruses. In 2015, A/Hunan/42443/2015 virus was isolated from a child in China with a severe infection. Molecular analysis revealed that this virus possessed several key virulence and human adaptation markers. Similar to what was previously observed in C57BL/6J mice, we report here that in the BALB/c mouse model, A/Hunan/42443/2015 virus caused more severe morbidity and higher mortality than did North American variant H1 virus isolates. Furthermore, the virus efficiently replicated throughout the respiratory tract of ferrets and exhibited a capacity for transmission in this model, underscoring the need to monitor zoonotic viruses that cross the species barrier as they continue to pose a pandemic threat.

## 1. Introduction

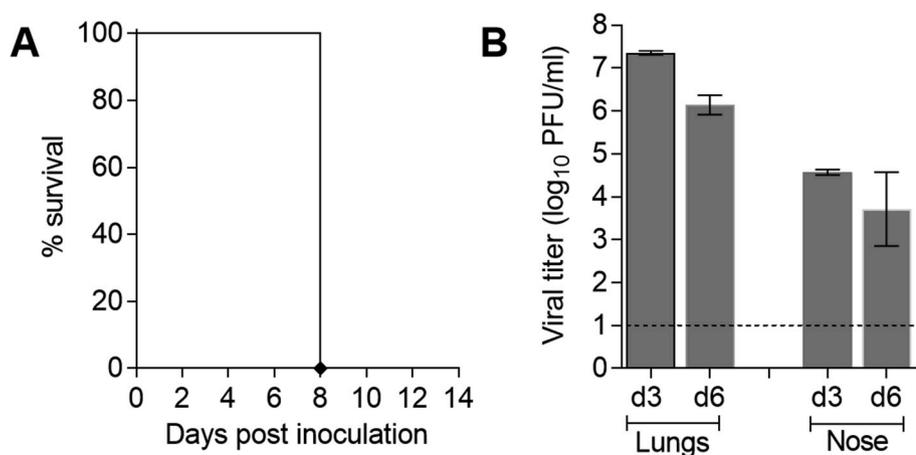
Influenza A virus (IAV) is an important respiratory pathogen that continually causes a substantial public health burden worldwide. With respect to H1 subtype viruses, three genetic lineages of IAV currently circulate in swine. The first swine lineage, referred to as the classical swine lineage, likely emerged prior to the 1918 H1N1 Spanish flu pandemic virus (Smith et al., 2009). The second lineage endemic in swine, human seasonal-like, resulted from independent introductions of human seasonal influenza viruses into swine populations in Europe, North America, and South America. The third lineage, Eurasian avian-like (EA), resulted from introduction of avian influenza in swine in Europe which subsequently spread into Asia (Anderson et al., 2016). Swine influenza viruses occasionally cross the species barrier and infect humans. These isolates are uniquely referred to as variant influenza viruses and are identified by adding a letter “v” to the virus subtype (CDC, 2018). Zoonotic infections are of concern from a public health standpoint as variant influenza viruses often are antigenically divergent from seasonal influenza viruses, hence may not be covered by seasonal vaccination (WHO, 2017). Several H1v infections have been reported in North America, Europe, and Asia (Myers et al., 2007; Shu et al., 2012; Zhu et al., 2016; Wang et al., 2013; de Jong et al., 1988; Yang et al., 2012; Qi et al., 2013; Shinde et al., 2009; Winter et al., 2013; Pulit-

Penaloza et al., 2018a, 2018b). A cycle of human-to-swine transmission, subsequent evolution by drift and shift in swine, followed by swine-to-human transmission is evident as many of the recently isolated swine viruses, including variant strains, contained genes of 2009 H1N1 pandemic (H1N1pdm09) viruses (Pulit-Penaloza et al., 2018a, 2018b; Sun et al., 2016; Qiao et al., 2014; He et al., 2018).

EA swine H1N1 virus was first detected in 1979 in Europe. Since then, it has been circulating in multiple Eurasian countries while occasionally causing human infections (Myers et al., 2007; Wang et al., 2013; Yang et al., 2012; Qi et al., 2013). The dominant EA swine lineage viruses in China have evolved through genetic reassortment and drift into several genotypes (Yang et al., 2016). In 2015, a novel reassortant influenza H1N1v virus, A/Hunan/42443/2015 (Hunan/42443), was isolated from a 30-month-old boy in Hunan Province, China. The child developed severe complications including pneumonia, respiratory failure, acute respiratory distress syndrome, and heart failure, before recovering from infection. Despite no evidence of human-to-human transmission (Zhu et al., 2016), pooled human sera poorly reacted with the virus and a candidate vaccine virus was generated for this virus strain (WHO, 2017). The virus contained EA H1N1 lineage HA, NA, and M genes, H1N1pdm09 origin PB1, PB2, PA and NP genes, and a classical swine lineage H1N1 NS gene. Hunan/42443 virus displayed higher infectivity and virulence in a C57BL/6J mouse model

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**Fig. 1. Pathogenicity of A/Hunan/42443/2015 A(H1N1v) influenza virus in BALB/c mice.** Eleven mice were inoculated with 5.0 log<sub>10</sub> PFU of Hunan/42443 virus. Five mice were monitored for signs of morbidity and mortality for 14 days p.i.; percent survival is shown (A). Lung and nose tissues were collected from the remaining mice for titer determination by plaque assay on days 3 (n = 3) and 6 (n = 3) p.i. (B). Mean titers are expressed as log<sub>10</sub> PFU/ml ± SD. The limit of detection is 10 PFU (dashed line).

as compared to recently isolated North American H1N1v viruses (Zhu et al., 2016). In this study, we evaluated pathogenesis and transmissibility of this virus using BALB/c mice and ferrets. Hunan/42443 virus replicated efficiently in BALB/c mice without prior adaptation and resulted in 100% mortality, unlike other H1v viruses, which typically possess mild virulence in this species (Pulit-Penaloza et al., 2018a). Replication was also evident throughout the respiratory tract of ferrets and the virus was readily transmitted among co-housed ferrets but transmitted less efficiently through the air. Our findings highlight the pandemic potential and public health risk associated with swine influenza viruses that are capable of jumping the species barrier to cause human infections.

## 2. Materials and methods

**Viruses.** Virus stocks of A/Hunan/42443/2015 H1N1v virus were propagated in MDCK cells at 37 °C for 48 h (Balish et al., 2013). Pooled cell supernatants were clarified by centrifugation, and frozen in aliquots at -80 °C. Virus titers in each stock were determined by standard plaque assay in MDCK cells (Maines et al., 2005). The stock viruses were sequenced and real-time RT-PCR exclusivity tests were performed to ensure no contamination of other subtypes of influenza A viruses. All work was conducted in a BSL3-enhanced laboratory setting.

**Mouse experiments.** All animal experiments were performed under the guidance of the Centers for Disease Control and Prevention's Institutional Animal Care and Use Committee and were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility. Eleven 6–8 week old female BALB/c mice (Charles River Laboratories, Wilmington, MA) were anesthetized intraperitoneally with 0.2 ml of 2,2,2-tribromoethanol in tert-amyl alcohol (Avertin; Acros Organics) and inoculated intranasally (i.n.) with 50 μl of 5 log<sub>10</sub> plaque forming units (PFU) of Hunan/42443 virus diluted in phosphate-buffered saline (PBS). Five mice were monitored for 14 days post-inoculation (p.i.) for clinical signs of infection and loss of body weight. On days 3 and 6 p.i., 3 mice each were euthanized for determination of viral titers in nose and lung tissues by plaque assay (Maines et al., 2005). Mice that lost ≥25% of pre-inoculation body weight were humanely euthanized.

**Ferret experiments.** Six-month old male Fitch ferrets (Triple F Farms, Sayre, PA), serologically negative for currently circulating influenza viruses, were housed in Duo-Flo Bioclean mobile units (Lab Products Incorporated, Seaford, DE) during experimentation. Six ferrets were inoculated i.n. with 6 log<sub>10</sub> PFU of Hunan/42443 virus diluted in 1 mL of PBS. The following day, contact was established by placing a serologically naive ferret in the same cage as each inoculated ferret (three ferret pairs), and respiratory droplet contact was established by placing a naive ferret in a cage with a perforated side-wall adjacent to the same type cage housing an inoculated ferret (three ferret pairs)

(Maines et al., 2006). Clinical signs of infection were monitored for 14 days p.i. and nasal wash samples were collected every two days from inoculated and contact ferrets for virus titer determination. Three additional inoculated ferrets were euthanized on day 3 p.i. for the assessment of virus replication in tissues as previously described (Maines et al., 2005). Convalescent sera collected from all surviving ferrets 3 weeks p.i. or post-contact (p.c.) were tested by hemagglutination inhibition assay using homologous virus and 0.5% turkey red blood cells to determine seroconversion (Stephenson et al., 2003).

## 3. Results

The Hunan/42443 H1N1v virus was previously shown to replicate efficiently in C57BL/6J mouse lungs without prior adaptation and to cause fatal infection at high inoculum doses (5–6 log<sub>10</sub> TCID<sub>50</sub>) (Zhu et al., 2016), suggesting enhanced virulence of this H1N1v virus compared to most North American variant influenza viruses and EA lineage swine isolates, which were previously tested in BALB/c mice (Pulit-Penaloza et al., 2018a; He et al., 2018; Yang et al., 2016; Belser et al., 2010). Because there are genetic differences and immune variations among inbred mouse strains that have the potential to affect the responses to influenza virus infection (Sellers et al., 2012), we chose to build upon previous findings by characterizing Hunan/42443 virus using a BALB/c mouse model. The mice were inoculated i.n. with 50 μl of 5 log<sub>10</sub> PFU of virus and assessed for morbidity, mortality, and viral replication. In agreement with the data in C57BL/6J mice, all infected BALB/c mice exhibited pronounced weight loss by day 8 p.i. and were euthanized (Fig. 1A). The virus replicated efficiently and to high titers throughout the murine respiratory tract. During the acute phase of infection, the average titers were 7.4 log<sub>10</sub> PFU/ml on day 3 p.i. and 6.1 log<sub>10</sub> PFU/ml on day 6 p.i. in mouse lungs, and 4.6 log<sub>10</sub> PFU/ml on day 3 p.i. and 3.7 log<sub>10</sub> PFU/ml on day 6 p.i. in nose tissue (Fig. 1B). The majority of previously tested North American variant influenza viruses and EA lineage swine isolates did not cause mortality in BALB/c mice, with a few exceptions where ≤80% mortality was observed (Pulit-Penaloza et al., 2018a; He et al., 2018; Yang et al., 2016; Belser et al., 2010). Our findings indicate enhanced virulence of Hunan/42443 virus in the BALB/c mouse model, compared to previous isolates.

Unlike mice, ferrets offer many advantages as a small mammalian model for the study of influenza viruses, including close physiologic similarity of human and ferret respiratory tracts, distribution of sialic acid receptors, and generally comparable clinical signs and transmissibility of influenza virus as in humans. The use of the ferret model has been indispensable in studies to evaluate the pandemic potential of novel influenza viruses (Belser et al., 2016). Ferrets that were inoculated i.n. with 6 log<sub>10</sub> PFU of Hunan/42443 virus exhibited moderate signs of infection, including weight loss (13.5% mean maximum), transient fever (1.5 °C mean rise above baseline), and the majority of

**Table 1**  
Pathogenicity and transmission of A/Hunan/42443/2015 A (H1N1v) virus in ferrets.

Ferret group <sup>a</sup>	Virus in NW <sup>b</sup>		Seroconversion <sup>c</sup>		% Weight loss <sup>d</sup>	Fever (°C) <sup>e</sup>	Nasal discharge	Sneezing
	# ferrets	Virus titer	# ferrets	HI titer				
Inoculated	6/6	5.8 ± 0.8	6/6	320-2560	13.5 (6/6)	1.5 (6/6)	6/6	4/6
Contact (DCT)	3/3	5.9 ± 0.6	3/3	1280-2560	7.5 (3/3)	1.2 (3/3)	2/3	0/3
Contact (RDT)	2/3	6.2 ± 0.9	2/3	640-1280	11.6 (2/3)	1.5 (2/3)	1/3	0/3

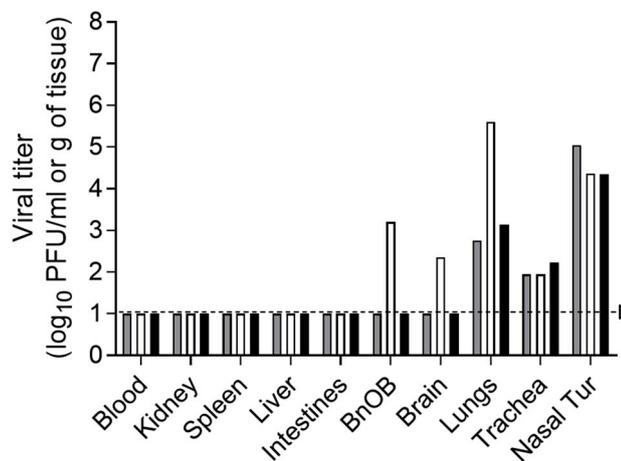
<sup>a</sup> Ferrets were intranasally inoculated or exposed to virus in a direct contact transmission (DCT) model or a respiratory droplet transmission (RDT) model.

<sup>b</sup> Number of ferrets with detectable virus in nasal wash (NW) samples and mean peak titer ( $\log_{10}$  PFU/ml) ± SD is shown.

<sup>c</sup> Number of ferrets that seroconverted by day 20 (contact ferrets) and 21 (inoculated ferrets) to homologous virus in a hemagglutination inhibition (HI) assay.

<sup>d</sup> Percentage mean maximum weight loss is shown with the number of ferrets displaying weight loss in parentheses.

<sup>e</sup> Mean maximum rise in temperature relative to baseline (38.4–39.3 °C) is shown with the number of ferrets displaying fever in parentheses.



**Fig. 2.** Detection of A/Hunan/42443/2015 A(H1N1v) influenza virus in ferret tissues. Viral titers in tissues collected on day 3 post inoculation with 6.0  $\log_{10}$  PFU of virus. Blood and nasal turbinate (Nasal Tur) viral titers are presented as  $\log_{10}$  PFU/ml and kidney, spleen, liver, intestines (pooled duodenum, jejunum-ileum loop, and descending colon), olfactory bulb (BnOB), brain (pooled anterior and posterior brain), lungs (each lobe sampled and pooled), and trachea are presented as  $\log_{10}$  PFU/g of tissue. Bars represent individual ferrets and are expressed as  $\log_{10}$  PFU/ml. The limit of detection is 10 PFU (dashed line).

inoculated ferrets displayed nasal discharge and sneezing (Table 1). Similar to what was observed in mice, Hunan/42443 virus was found throughout the ferret respiratory tract on day 3 p.i., with mean titers of 4.6  $\log_{10}$  PFU/ml in nasal turbinates, 2.0  $\log_{10}$  PFU/g in tracheal tissue and 3.8  $\log_{10}$  PFU/g in lung tissue (Fig. 2). Virus was not detected outside of the respiratory tract, except for the detection of infectious virus in the olfactory bulb and brain of 1 out of 3 ferrets. The viral load and distribution of Hunan/42443 virus in tissues was comparable with North American H1N1v influenza viruses and Asian swine isolates tested in the ferret model (Pulit-Penaloza et al., 2018a; Yang et al., 2016).

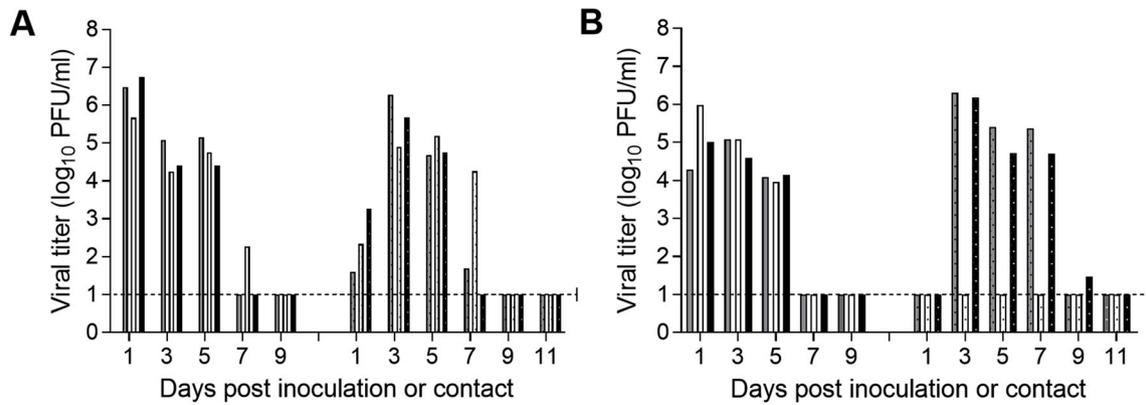
North American H1N1 swine-origin influenza viruses have been shown to efficiently transmit among cohoused ferrets, but with varying efficiency through the air via respiratory droplets or droplet nuclei (Pulit-Penaloza et al., 2018a, 2018b; Barman et al., 2012). Similarly, Asian swine influenza isolates have been shown to possess the ability to transmit among ferrets via air with varying efficiencies (Yang et al., 2016; Pascua et al., 2012), but transmissibility of Hunan/42443 virus in ferrets has not been previously tested. Transmission experiments were performed by housing a naïve ferret in the same cage as an inoculated ferret to test transmission between animals that are in direct contact (3 ferret pairs). This model provides the opportunity for transmission to occur via contact or inhalation (Fig. 3A). Transmission experiments were also performed by placing a naïve ferret in an adjacent cage allowing for air exchange through perforated cage side walls (3 ferret

pairs) which prevents transmission from occurring due to direct or indirect contact (Fig. 3B). Nasal wash specimens were collected every other day from both inoculated and contact ferrets to measure the level of infectious virus. Hunan/42443 virus replicated well in all 6 inoculated animals; peak nasal wash titers observed on day 1 or 3 p.i. ranged from 5.0 to 6.7  $\log_{10}$  PFU/mL (Fig. 3, left side of panels). Efficient transmission was observed between ferrets that were in direct contact as evidenced by the presence of virus in nasal wash samples from all contact ferrets as early as day 1 p.c. and seroconversion on day 20 p.c. (Fig. 3A, Table 1). In the respiratory droplet transmission model, 2 out of 3 contact ferrets became productively infected and shed comparable levels of virus to those found in the inoculated ferrets (6.2  $\log_{10}$  compared to 5.8  $\log_{10}$  PFU/ml; Fig. 3B, Table 1). Seroconversion was observed 20 days p.c. only in the two animals that shed virus (Table 1). These findings indicate that, although Hunan/42443 virus displays enhanced virulence in the mouse model, it requires further adaptation to present a pandemic threat.

#### 4. Discussion

Pigs represent important hosts for influenza viruses with pandemic potential. Susceptibility of swine to both human and avian influenza viruses, and continuous virus evolution via both genetic reassortment and drift, results in the expansion of swine influenza virus diversity (Lewis et al., 2016). Swine influenza viruses that occasionally cross the species barrier and infect humans are of particular public health concern as they often are antigenically distinct from human seasonal viruses and can have a great impact on public health if they gain the ability to efficiently spread from person-to-person (CDC, 2018). In 2015, A/Hunan/42443/2015, an H1N1v virus that was isolated from a child with severe pneumonia, raised concern partly due to its ability to cause severe morbidity and mortality in the C57BL/6J mouse model (Zhu et al., 2016), similar to what was observed in BALB/c mice infected with reconstructed 1918 virus (Tumpey et al., 2005). In this study, we further characterized the Hunan/42443 virus by performing a risk assessment of the pathogenicity in a different mouse model. Due to genetic differences between mouse strains, C57BL/6J mice predominantly induce Th-1 (cellular), while BALB/c mice induce Th-2 (humoral) immune responses; the severity of influenza virus infection differs for some virus strains depending on the mouse model used (Otte and Gabriel, 2011). Despite the differences in mouse strains, our findings in the BALB/c model were in agreement with previous observations in C57BL/6J mice; as mice inoculated i.n. with a comparable dose (5  $\log_{10}$  PFU) of Hunan/42443 virus displayed enhanced morbidity including ruffled fur, hunched posture, and severe weight loss and were humanely euthanized on day 8 post inoculation. Overall, the magnitude of viral replication and mortality rates in BALB/c mice inoculated with Hunan/42443 virus were higher as compared to other North American and EA lineage swine H1N1 isolates described previously (Pulit-Penaloza et al., 2018a, 2019; He et al., 2018).

We found that the clinical signs and symptoms of infection



**Fig. 3. Transmissibility of A/Hunan/42443/2015 A(H1N1v) influenza virus in ferrets.** Six ferrets were inoculated i.n. with 6.0 log<sub>10</sub> PFU of Hunan/42443 virus. The following day, a naive ferret was placed in each of the three cages housing an inoculated ferret for the direct contact transmission model (A) and in an adjacent cage to each of the 3 remaining animals for the respiratory droplet transmission model (B). Nasal wash specimens were collected from both the inoculated (left side of each panel) and contact ferrets (right side of each panel) every other day post inoculation or post contact for titration by plaque assay. Titers of individual ferrets are expressed as log<sub>10</sub> PFU/ml. The limit of detection is 10 PFU (dashed line).

displayed in ferrets inoculated with Hunan/42443 virus, including transient fever, weight loss, sneezing and rhinorrhea, along with the distribution of virus replication throughout the ferret respiratory tract, were comparable to previous reports for many swine-origin H1 viruses (Pulit-Penaloza et al., 2018a, 2018b; Yang et al., 2016; Barman et al., 2012). Unlike in mice, Hunan/42443 virus did not display enhanced virulence and did not cause mortality in ferrets. The virus transmitted between all three ferret pairs when placed in direct contact, with naive animals becoming infected within 24h of contact, demonstrating a high efficiency of transmission in this setting. However, in a more stringent respiratory droplet setting, the virus transmitted less efficiently, with 2 out of 3 contact ferrets shedding virus by day 3 p.c. These results indicate that Hunan/42443 virus possesses the ability to transmit between ferrets through the air, similar to North American swine influenza viruses isolated from humans (Pulit-Penaloza et al., 2019).

Surveillance for known genetic markers of mammalian adaptation can be helpful in predicting the pathogenicity, transmissibility, and public health risk associated with novel viruses. The PB2 subunit of the RNA polymerase complex is a major virulence and host range determinant of influenza viruses. Amino acid substitutions in PB2 including E627K, D701N, and G590S/Q591R have been demonstrated to be critical for increased polymerase activity, and enhanced viral replication and transmissibility in mammalian models (Steel et al., 2009; Liu et al., 2012; Mehle and Doudna, 2009; Van Hoeven et al., 2009). In general, the H1N1pdm09-origin PB2 gene lacks characteristic human/mammalian amino acid mutations, such as E627K and D701N, and rather possesses the compensatory amino acids 590S and 591R. The PB2 gene of Hunan/42443 virus is closely related to that of H1N1pdm09 virus (Zhu et al., 2016), the sequence (GISAID database ID, 206573) lacks 627K and 701 N markers, but possesses 591R, which could explain the efficient replication of Hunan/42443 virus in the mammalian models examined here. Two additional adaptation markers, T271A and T588I, are observed in the Hunan/42443 virus PB2 sequence. The T271A substitution was previously shown to increase polymerase activity in mammalian cells, leading to enhanced replication in mouse lungs and severe lung pathology (Bussey et al., 2010). In contrast, the T588I mutation, in addition to increased polymerase activity in mammalian cells, has been shown to mediate suppression of interferon-beta expression (Zhao et al., 2014). Although swine influenza viruses do not typically cause high mortality in mice, both the Hunan/42443 virus and a previously described swine H1N1v virus, A/Ohio/09/2015 (Pulit-Penaloza et al., 2018a), caused ≥80% mortality and both viruses contained 271A and 588I residues in the PB2 protein. This finding suggests that substitutions at these positions may play a role in the enhanced morbidity phenotype observed with Hunan/42443

virus in the murine model.

Each human infection caused by a swine-origin influenza virus provides another opportunity for these viruses to further adapt and become capable of sustained transmission in the human population; therefore, it is important to closely monitor novel swine influenza viruses to gain a greater understanding of the molecular adaptations necessary for sustained transmission in susceptible populations. EA lineage influenza viruses from zoonotic sources can spread readily to other continents, as recently demonstrated by the spread of H5Nx and canine influenza viruses (Pulit-Penaloza et al., 2015, 2017; Hon et al., 2015; Lee et al., 2017), underscoring the necessity to perform risk assessments of influenza viruses of multiple lineages from diverse geographic areas. Furthermore, the information in this and other similar studies aid in the characterization of emerging influenza viruses and in the generation of candidate vaccine viruses, which collectively contribute to the worldwide efforts for pandemic preparedness.

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