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Mammalian Pathogenesis and Transmission of H7N9 Influenza Viruses from Three Waves, 2013-2015

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ABSTRACT

Three waves of human infection with H7N9 influenza viruses have concluded to date, but only viruses within the first wave (isolated between March and September 2013) have been extensively studied in mammalian models. While second- and third-wave viruses remain closely linked phylogenetically and antigenically, even subtle molecular changes can impart critical shifts in mammalian virulence. To determine if H7N9 viruses isolated from humans during 2013 to 2015 have maintained the phenotype first identified among 2013 isolates, we assessed the ability of first-, second-, and third-wave H7N9 viruses isolated from humans to cause disease in mice and ferrets and to transmit among ferrets. Similar to first-wave viruses, H7N9 viruses from 2013 to 2015 were highly infectious in mice, with lethality comparable to that of the well-studied A/Anhui/1/2013 virus. Second- and third-wave viruses caused moderate disease in ferrets, transmitted efficiently to cohoused, naive contact animals, and demonstrated limited transmissibility by respiratory droplets. All H7N9 viruses replicated efficiently in human bronchial epithelial cells, with subtle changes in pH fusion threshold identified between H7N9 viruses examined. Our results indicate that despite increased genetic diversity and geographical distribution since their initial detection in 2013, H7N9 viruses have maintained a pathogenic phenotype in mammals and continue to represent an immediate threat to public health.

IMPORTANCE

H7N9 influenza viruses, first isolated in 2013, continue to cause human infection and represent an ongoing public health threat. Now entering the fourth wave of human infection, H7N9 viruses continue to exhibit genetic diversity in avian hosts, necessitating continuous efforts to monitor their pandemic potential. However, viruses isolated post-2013 have not been extensively studied, limiting our understanding of potential changes in virus-host adaptation. In order to ensure that current research with first-wave H7N9 viruses still pertains to more recently isolated strains, we compared the relative virulence and transmissibility of H7N9 viruses isolated during the second and third waves, through 2015, in the mouse and ferret models. Our finding that second- and third-wave viruses generally exhibit disease in mammals comparable to that of first-wave viruses strengthens our ability to extrapolate research from the 2013 viruses to current public health efforts. These data further contribute to our understanding of molecular determinants of pathogenicity, transmissibility, and tropism.

Since their initial detection in humans in the spring of 2013, low-pathogenicity avian influenza (LPAI) H7N9 viruses have represented a continued public health threat in Southeast Asia, with over 700 laboratory-confirmed cases of human infection to date (1). Human cases have been limited to China or among travelers who visited China before returning to their home country (2). Asymptomatic H7N9 virus-infected chickens appear to be central to the persistence and expansion of this outbreak (3); accordingly, poultry contact and visitation of live poultry markets has been linked with H7N9 virus infection (4, 5), and the closure of live poultry markets has been associated with a decline of new human infections in 2013 and 2014 (6, 7). Limited family clusters of H7N9 virus infection have been reported (8, 9), but human-to-human transmission has remained a rarely documented and unsustainable event (10), while human infections continue to occur following exposure to H7N9 viruses circulating in avian reservoirs (11).

To date, three completed epidemiologic waves of human cases with H7N9 viruses have taken place in Southeast Asia (9). The first wave of human infection (30 March to 30 September 2013) resulted in over 130 confirmed cases with a >30% fatality rate, with human infection detected in mainland China and Thailand. The second wave (1 October 2013 to 30 September 2014) yielded over 260 additional cases, with the majority of cases detected in Guangdong and Zhejiang provinces of China (9) and additional cases detected in Hong Kong, Thailand, and Malaysia. The third wave (1 October 2014 to 30 September 2015) resulted in over 220 reported cases in China, Hong Kong, and Canada. Disease symptoms, severity, and mortality generally have held constant between all three waves, and no pronounced antigenic drift has been detected from 2013 to 2015 (2). However, increased case-fatality rates re-

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TABLE 1 LPAI H7N9 viruses used in this study

Virus name	Abbreviation	Wave	Patient outcome	Reference or source
A/shoveler/Egypt/00215-NAMRU3/2007	Shv/Egypt/07	NA[a]	NA	61
A/Anhui/1/2013	Anhui/1/13	First	Fatal	45
A/Taiwan/1/2013	Taiwan/1/13	First	Survived	62
A/Hong Kong/5942/2013	HK/5942/13	Second	Survived	This study
A/Taiwan/1/2014	Taiwan/1/14	Second	Survived	38
A/Taiwan/2/2014	Taiwan/2/14	Second	Survived	38
A/Hong Kong/734/2014	HK/734/14	Second	Fatal	This study
A/Hong Kong/56/2015	HK/56/15	Third	Survived	This study
A/British Columbia/1/2015	BC/1/15	Third	Survived	63

[a] NA, not applicable.

ported in one province in China between cases from the first and second waves suggested that despite high homology between viruses isolated from different waves, it was possible that H7N9 viruses developed an enhanced ability to cause disease in humans (12). Previous studies with H7 subtype avian influenza viruses have demonstrated that even subtle changes can indicate enhanced adaptation to mammals, including increased transmissibility among mammalian hosts (6, 13–15), underscoring the need for ongoing surveillance and characterization of emerging H7N9 viruses as they continue to cause human infections.

The majority of published studies examining H7N9 viruses have focused on first-wave viruses only. However, as H7N9 viruses continue to cause human infection, the increased temporal, geographic, and genetic distribution of these viruses necessitates continued investigation (11). Notably, second- and third-wave viruses have not been extensively examined *in vitro* or *in vivo*, and the contributing role of precursor viruses to H7N9 pathogenicity is still understudied (16). Here, we characterized the mammalian pathogenicity and transmissibility of first-, second-, and third-wave H7N9 viruses. We found that H7N9 viruses possess comparable virulence in the mouse and ferret over three waves of human infection. Similar to viruses from the first wave, H7N9 viruses isolated from the second and third waves maintain transmissibility in the ferret model in the presence of direct contact and exhibit limited transmissibility by respiratory droplets. Generally comparable replication kinetics in human respiratory tract cells and the pH of fusion between viruses from all waves indicates that H7N9 viruses continue to remain a pandemic threat.

MATERIALS AND METHODS

Viruses. H7N9 influenza A viruses used in this study are shown in Table 1. Virus stocks were propagated in the allantoic cavity of 10-day-old embryonated hens' eggs at 37°C for 42 h. Allantoic fluid from multiple eggs was pooled, clarified by centrifugation, and frozen in aliquots until use at −80°C. Virus stocks were tested by standard plaque assay in Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) for determination of PFU titer (17). All experiments were conducted under biosafety level 3 containment, including enhancements as required by the U.S. Department of Agriculture and the National Select Agent Program (18).

Ethics statement. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Centers for Disease Control and Prevention and were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility.

Mouse experiments. Female BALB/c mice (Jackson Laboratories), 6 to 8 weeks of age, were anesthetized with 2,2,2-tribromoethanol in *tert*-amyl alcohol (Avertin; Sigma-Aldrich, St. Louis, MO). Mice were inoculated with 10^6 PFU of virus by either the intranasal (i.n.) route in a 50-μl volume diluted in phosphate-buffered saline (PBS) or the ocular (i.o.) route, with 5 μl of virus diluted in PBS dropped onto the corneal surface of the right eye and massaged in by using the eyelids following corneal scarification as described previously (19). The 50% mouse infectious dose (MID_{50}) and 50% lethal dose (LD_{50}) were determined by inoculating mice i.n. with 10-fold serial dilutions of virus. Five mice per group were monitored daily for 14 days for morbidity and to obtain the LD_{50}, and 3 mice were euthanized day 3 postinoculation (p.i.) for the collection of lungs for titration to obtain the MID_{50} for each virus. Any mouse that lost >25% of preinfection body weight was euthanized. The replication and systemic spread of each virus was determined by harvesting the eyes, nose, lung, and brain of mice ($n = 3$) on days 3 and 6 p.i., with tissues titrated by standard plaque assay (limit of detection, 10 PFU).

Ferret experiments. Male Fitch ferrets (Triple F Farms), 6 to 8 months of age and serologically negative by standard hemagglutination inhibition for currently circulating influenza viruses, were used in this study. Ferrets were housed in a Duo-Flo Bioclean mobile environmental enclosure (Lab Products, Seaford, DE) for the duration of each experiment. Ferrets were inoculated i.n. with 10^6 PFU of each indicated virus in a 1-ml volume diluted in PBS and observed daily for clinical signs and symptoms of infection, with nasal washes (NW), conjunctival washes (CW), and rectal swabs (RS) collected on alternate days p.i. to measure virus shedding as previously described (20). Three additional ferrets inoculated with 10^6 PFU of each virus were euthanized day 3 p.i. for the assessment of virus replication and systemic spread as previously described (21). All samples were titrated by standard plaque assay. For histopathological analyses, respiratory tissues from animals euthanized day 3 p.i. were fixed in 10% neutral buffered formalin and embedded in paraffin. Four-micrometer sections from formalin-fixed, paraffin-embedded specimens were stained with hematoxylin and eosin (H&E) for histopathologic evaluation.

Virus transmissibility was assessed by placing a naive ferret in the same cage as an inoculated ferret (to assess transmission in the presence of direct contact [DC]) or in an adjacent cage with modified side walls to allow air exchange in the absence of direct or indirect contact between animals (to assess transmission by respiratory droplets [RD]) as previously described (22). Serum was collected on days 17 to 23 p.i./postcontact (p.c.) to measure seroconversion to homologous virus.

Cell culture and viral replication. The human bronchial epithelial cell line Calu-3 (ATCC) was grown on membrane inserts and cultured as previously described (17). Cells were grown to confluence on 6-well inserts for 1 week to achieve a transepithelial resistance of >1,000 Ω. To measure replication kinetics, virus was added apically in serum-free medium to cells at a multiplicity of infection (MOI) of 0.01 and incubated 1 h before washing and culturing for the duration of the experiment at 37°C or 33°C. Aliquots of culture supernatant at the indicated times p.i. were immediately frozen at −80°C until titration for the presence of infectious virus by standard plaque assay. The statistical significance of replication kinetics in Calu-3 cells was determined by 2-way analysis of variance (ANOVA) with a Bonferroni posttest.

TABLE 2 Amino acid substitutions in the proteins of three waves of H7N9 viruses

		Substitution in:										
		HA[a]					PB2					PA
Virus	Wave	57	132	186	226	276	191	559	570	627	701	394
Anhui/1/13	First	R	T	V	L	N	K	N	M	K	D	N
Taiwan/1/13	First	R	T	V	P	N	K	N	M	K	D	N
HK/5942/13	Second	R	A	V	L	N	E	T	I	K	D	N
Taiwan/1/14	Second	R	T	V	L	N	E	T	I	K	D	D
Taiwan/2/14	Second	R	T	V	L	N	E	T	I	E	N	N
HK/734/14	Second	K	A	V	L	N	E	T	I	K	D	D
HK/56/15	Third	K	T	A	L	D	E	T	M	K	D	D
BC/1/15	Third	R	T	V	L	N	E	T	M	K	D	D

[a] H3 numbering.

pH threshold for influenza virus fusion activation. The influenza virus-induced hemolysis assay was carried out as a surrogate assay to measure the pH threshold for H7 influenza virus fusion activation as described previously (23). Briefly, 50 μl of H7 viruses in triplicate containing 128 hemagglutinin (HA) units or PBS mock-treated control were incubated with 50 μl of 1% (vol/vol) turkey red blood cells (TRBC) in 96-well plates at 4°C for 1 h. The virus and TRBC mixture was pelleted and resuspended in 150 μl of citric fusion buffer (20 mM HEPES, 2 mM $CaCl_2$, 150 mM NaCl, 20 mM citric acid monohydrate/sodium citrate tribasic dehydrate) at various pH values at 37°C for 1 h to induce hemolysis. The released hemoglobin resulting from virus fusion with TRBC was measured by the optical density (OD) at 405 nm after removing nonlysed TRBC pellets at 2,000 rpm for 5 min and subtracting the background OD value from the PBS mock control at each pH value. The maximal hemolysis at a given pH was normalized to 100% and the hemolysis at other pH values was expressed as a percentage of the maximal hemolysis; results were plotted as averages from three independent experiments.

RESULTS

H7N9 virus information and genetic diversity. To study the mammalian pathogenicity and transmissibility of H7N9 viruses from 2013 to 2015, we first identified representative viruses associated with human infection from each of the first three waves (Table 1). All human isolates tested in this study were likely the result of independent avian exposures to the virus and originated from different geographical areas. All patients infected with the indicated viruses exhibited respiratory symptoms, ranging from mild (i.e., influenza-like illness) to severe (i.e., acute respiratory distress syndrome); two isolates were from fatal cases. A representative precursor avian H7N9 virus (shv/Egypt/07) was included in the study to better contextualize phenotypic changes associated with mammalian adaptation.

Since their first detection in 2013, the spread of H7N9 viruses throughout China has led to the establishment of multiple lineages and virus genotypes (3). The spread of H7N9 viruses throughout China and potential ongoing reassortment with circulating H9N2 viruses had led to the establishment of a diverse array of viral genotypes (24, 25). Sequence alignments of the viruses isolated from the second and third waves revealed several differences from the well-studied first-wave Anhui/1 virus (3, 24). While most of these differences were present only in selected isolates and were not consistent across viruses from each epidemic wave, selected mutations (most notably in the polymerase genes) did appear to become more common in the second and/or third waves of the epidemic. Nonetheless, the internal genes of all viruses isolated from humans described here maintained greater than 99% amino acid identity with Anhui/1/13 virus (data not shown). Table 2 highlights these residues as well as those in the hemagglutinin that are believed to influence antigenicity and receptor binding. As is typical of most H7N9 human isolates, seven of the eight human-derived H7N9 viruses included in this study possess a lysine at PB2 position 627. Taiwan/2/14 virus bears a glutamic acid at position 627 but features a 701 asparagine residue, which, like 627, is associated with mammalian adaptation in this subtype (26). Similarly, with the exception of one first-wave isolate, all viruses in this study maintain a leucine at position 226 (H3 numbering) of the hemagglutinin, which is also associated with mammalian host adaptation (27). Mutations in the HA and polymerase genes between viruses isolated from the first, second, and third waves prompted us to choose representative viruses from each wave for further characterization in *in vitro* and *in vivo* models.

Pathogenesis of first-, second-, and third-wave H7N9 viruses in mice. H7N9 viruses isolated from the beginning of the H7N9 outbreak in 2013 were found to cause moderate to severe disease in mice (28–32), but viruses isolated from 2014 to 2015 have not been examined as extensively. We studied the pathogenicity of selected first (Taiwan/1/13)-, second (HK/5942/13)-, and third (BC/1/15)-wave viruses in the BALB/c mouse as they compared to the well-characterized, first-wave Anhui/1 virus (Table 3). Regardless of the wave of isolation, all H7N9 viruses tested exhibited a high degree of infectivity and comparable virulence in this species, leading to severe (≥20%) weight loss and pronounced mortality. Viral titers on days 3 and 6 p.i. were high in both the lung (≥$\log_{10}$ 5.5 PFU/ml) and nose (≥$\log_{10}$ 2.2 PFU/ml), comparable to H5N1 viruses, which cause similar morbidity and mortality in mice (21). However, viral titers in the brain were not detected for any virus on day 6 p.i. (data not shown).

Unlike most H7 subtype viruses, H7N9 viruses have not been associated with ocular disease in humans. However, as the eye represents a secondary route of virus entry to mount a respiratory infection, we examined the ability of H7N9 viruses from three waves to infect mice following ocular inoculation (Table 4). As reported previously and confirmed here, mice inoculated i.o. with Anhui/1 virus possessed detectable virus in the nose, but it was detected only sporadically in the eye or lung day 3 or 6 p.i. (16, 28). However, Taiwan/1/13, HK/5942/13, and BC/1/15 viruses exhibited the capacity to replicate to detectable titers in all three tissues following i.o. inoculation. These findings indicate that while

TABLE 3 Mouse infectivity and tissue viral titers of H7N9 viruses

Virus	% wt loss[a] (day p.i.)	Infectivity[b]		Viral titer by day p.i.[c]			
				3		6	
		MID_{50}	LD_{50}	Lung	Nose	Lung	Nose
Anhui/1[d]	22.2 (7)	0.25	3.4	7.2 ± 0.6	3.2 ± 0.4	5.5 ± 0.5	2.2 ± 0.7
Taiwan/1/13	27.4 (5)	0.25	3.5	7.4 ± 0.1	5.0 (1/3)[e]	6.9 ± 0.6	2.4 ± 0.2
HK/5942/13	19.6 (5)	0.10	3.2	7.7 ± 0.1	4.2 ± 0.3	7.0 ± 0.3	3.1 ± 0.5
BC/1/15	22.4 (5)	0.25	2.8	7.3 ± 0.6	3.6 ± 0.2	7.3 ± 0.1[f]	3.2 ± 0.2[f]

[a] Mean maximum percent weight loss (5 mice per group) following inoculation with 10^6 PFU.
[b] Fifty percent mouse infectious dose (MID_{50}) and 50% lethal dose (LD_{50}) are expressed as the $\log_{10}$ PFU required to give one MID_{50} or one LD_{50}, respectively.
[c] Virus endpoint titers are expressed as the mean $\log_{10}$ PFU/ml ± SD from 3 mice per tissue. The limit of virus detection was 10 PFU.
[d] Includes data published previously in reference 28.
[e] Virus was detected in only 1 of 3 inoculated mice.
[f] Due to mice not surviving to day 6 p.i. with BC/1/15 virus, titers reflect two mice inoculated with 10^6 PFU and one mouse inoculated with 10^5 PFU.

H7N9 viruses from three waves do not maintain the ocular tropism observed with H7 viruses, they nonetheless possess the ability to use the eye as a portal of entry in mammals.

Pathogenesis of first-, second-, and third-wave H7N9 viruses in ferrets. The presence of amino acid changes among H7N9 viruses indicative of mammalian adaptation warrants the examination of recently isolated H7N9 viruses in multiple mammalian models (33). We first examined the precursor LPAI H7N9 virus shv/Egypt/07 in ferrets, which shares high surface glycoprotein amino acid identity with Anhui/1/13 virus and was previously shown to exhibit reduced virulence in the mouse model compared with human isolates from 2013 (28). Ferrets inoculated with this avian virus exhibited mild weight loss (<3%), and virus was detected only in respiratory tract tissues, with no extrapulmonary spread to ocular, intestinal, or brain tissues (Table 5), comparable to other LPAI viruses isolated from avian species in this model (30).

The mild to moderate disease in ferrets caused by infection with first-wave H7N9 viruses has been established (28, 30, 31, 34, 35). We next examined if H7N9 viruses isolated from the second and third waves exhibited virulence comparable to that of first-wave viruses in the ferret model. Infection with all H7N9 viruses examined caused a transient rise in body temperature in ferrets, ranging from 1.6 to 1.9°C above baseline (Table 5). Second- and third-wave viruses (HK/5942/13 and BC/1/15) induced somewhat greater weight loss in ferrets compared to first-wave H7N9 viruses (Anhui/1/13 and Taiwan/1/13). All H7N9 viruses isolated from humans replicated efficiently throughout the ferret respiratory tract, reaching mean peak titers ranging from $\log_{10}$ 5.4 to 6.2 PFU/ml in nasal turbinates and $\log_{10}$ 5.0 to 6.5 PFU/g in lung tissues. Virus was detected sporadically and at low titer ($\leq$2.4 $\log_{10}$ PFU/ml or g) in ocular and intestinal samples. First-wave H7N9 viruses were detected in the olfactory bulb or brain of 1 out of 6 ferrets (Table 5) (28), while second- and third-wave viruses were detected in these tissues more frequently but at titers comparable to those of the first-wave viruses.

Histopathologic evaluation of tissues collected day 3 p.i. from H7N9 virus-infected ferrets revealed a spectrum of inflammation depending on the virus used to infect, although striking differences between H7N9 isolates were not observed (Fig. 1). Airway tissues showed minimal to moderate tracheobronchitis (Fig. 1A to E), while the lung tissues show mild to severe bronchopneumonia (Fig. 1G to K) in different groups of infected animals. The most severe inflammation was observed in Anhui/1 virus-infected ferrets (Fig. 1A and G), whereas infection with the seasonal H3N2 virus A/Texas/50/12 (28) resulted in reduced inflammation in these tissues (Fig. 1F and L). Together, these data suggest that second- and third-wave H7N9 viruses generally have maintained the moderate virulence observed with the first-wave H7N9 virus.

Transmissibility of first-, second-, and third-wave H7N9 viruses in ferrets. Avian H7N9 viruses isolated before or concurrent with the first detection of human cases have demonstrated no or only limited transmission in the ferret model by either respiratory droplets or in the presence of direct contact (30, 31, 36). In contrast, H7N9 viruses isolated in 2013, during the first wave of the outbreak, demonstrated the capacity for efficient transmission between cohoused ferrets and limited transmission in the more stringent respiratory droplet model (28, 30, 31, 34, 35, 37). To

TABLE 4 Replication of H7N9 influenza viruses following ocular inoculation in mice

Tissue	Day p.i.	Virus titer[a]			
		Anhui/1/13[b]	Taiwan/1/13	HK/5942/13	BC/1/15
Eye	3	1.5 (1/7)	2.1 ± 0.1	2.9 ± 0.3	1.9 ± 0.5 (2/4)
Nose	3	2.7 ± 0.9 (4/7)	3.9 ± 0.8	2.3 ± 0.5	2.5 ± 0.4 (3/4)
Lung	3	0/7	2.1 (1/3)	1.4 ± 0.1	2.2 ± 0.7 (3/4)
Eye	6	2.1 ± 1.2 (2/7)	2.1 ± 0.5	2.2 ± 0.1	1.4 ± 0.2 (3/4)
Nose	6	2.8 ± 0.8 (6/7)	2.8 ± 0.6	2.7 ± 1.2	3.4 ± 0.2
Lung	6	1.6 (1/7)	1.5 (2/3)	5.1 ± 0.5 (2/3)	2.0 ± 0.6 (3/4)

[a] Mean virus titers in mice inoculated with 10^6 PFU/5 μl of virus following corneal scarification. Virus titers are expressed as the mean $\log_{10}$ PFU/ml ± SD among mice with positive virus detection (data are representative of all mice examined unless denoted in parentheses, in which the number of mice with positive virus detection/total number of mice in the group is indicated). The limit of virus detection was 10 PFU.
[b] Includes data published previously in reference 28.

TABLE 5 Replication of H7N9 influenza viruses in ferrets

Characteristic	Value(s) for:				
	Shv/Egypt/07	Anhui/1/13[f]	Taiwan/1/13	HK/5942/13	BC/1/15
Clinical[a]					
Fever[b]	1.6	1.6	1.9	1.8	1.8
wt loss[c]	2.2	8.8	4.4	15.1	11.3
Virus sample source and titer[d]					
CW[e]	0/3	6/11	1/3	3/3	6/6
RS[e]	0/3	7/11	1/3	0/3	1/6
Conj	0/3	1.8 (1/3)	2.2 ± 0.4 (2/3)	0/3	1.8 ± 0.4
Int	0/3	0/3	1.7 ± 0.5 (2/3)	1.6 (1/3)	2.4 (1/3)
Nas Tur	5.8 ± 0.4	6.2 ± 0.2	5.6 ± 0.6	5.4 ± 0.4	6.1 ± 0.6
Trachea	3.5 ± 0.6 (2/3)	5.5 ± 0.6	5.3 ± 0.8	3.3 (1/3)	5.1 ± 0.4
Lung	3.5 ± 2.2 (2/3)	5.6 ± 0.3	5.0 ± 0.9	5.0 ± 1.1	6.5 ± 0.1
OB	0/3	4.9 (1/3)	0/3	4.0 (1/3)	4.1 ± 2.1 (2/3)
Bn Ant	0/3	2.7 (1/3)	0/3	1.8 ± 0.7	3.8 ± 0.1 (2/3)

[a] Clinical signs, symptoms, and observations detected through 14 days p.i.
[b] Mean maximum rise in body temperature in degrees centigrade (baseline body temperature, 37.7 to 39.3°C).
[c] Percent mean maximum weight loss in ferrets.
[d] Unless specified otherwise, virus titers are expressed as the mean $\log_{10}$ PFU/g or ml ± SD among ferrets with positive virus detection on day 3 p.i. (data are representative of 3 ferrets examined, unless denoted in parentheses). The limit of virus detection was 10 PFU. Conj, surrounding conjunctival tissue; Int, pooled (duodenum, jejunum, and ileum) intestinal tissue; Nas Tur, nasal turbinates; OB, olfactory bulb; Bn Ant, anterior brain.
[e] Number of ferrets with detectable virus in conjunctival washes (CW) or rectal swabs (RS) collected on days 1, 3, and 5 p.i./total number of ferrets tested.
[f] Includes data published previously in reference 28.

determine if H7N9 viruses isolated since the first wave have maintained this transmission profile, ferrets were inoculated with selected H7N9 viruses from each of the first three waves. Twenty-four hours later, a naive ferret was placed in an adjacent cage that permits air exchange between ferret pairs but without direct or indirect contact. Consistent with previous findings, an avian precursor virus, shv/Egypt/07, did not transmit via respiratory droplets: no virus was detected in nasal washes, and no seroconversion to homologous virus was noted (Fig. 2A). Taiwan/1/13 virus differs in the M gene from Anhui/1/13 virus and does not possess a lysine at position 226 of the HA gene (38). While Anhui/1/13 virus previously was shown to transmit between members of 2 out of 6 ferret pairs (28), Taiwan/1/13 virus did not transmit to any ferrets by this route (Fig. 2B), highlighting strain-specific differences in virus transmissibility between first-wave H7N9 viruses. The second- and third-wave H7N9 viruses (HK/5942/13 and BC/1/15) demonstrated comparable transmissibility with Anhui/1/13 virus, with virus detected in nasal wash specimens of 1/3 ferrets for both viruses and seroconversion of 2/3 ferrets for HK/5942/13 virus and 1/3 ferrets for BC/1/15 virus by the end of the experiment (Fig. 2C and D). The efficient transmission of first-wave H7N9 viruses among cohoused animals (8/8 ferrets [28]) was maintained with both of the second- and third-wave H7N9 viruses tested here (Fig. 2E and F). Therefore, we can conclude that no enhancement in transmission capability occurred among the second- and third-wave H7N9 viruses evaluated here.

Replication of H7N9 viruses in human bronchial epithelial cells. First-wave H7N9 viruses isolated from the first human cases in 2013 demonstrated the ability to replicate efficiently in the human bronchial epithelial cell line Calu-3 at 37°C but less efficiently at a lower temperature (33°C) that is representative of the upper airways (28). To determine if H7N9 viruses from the second and third waves have maintained this property, we evaluated the replication kinetics of H7N9 viruses from all three waves in Calu-3 cells at both temperatures (Fig. 3). Titers of all eight H7N9 viruses examined were significantly lower at 33°C than at 37°C at both 12 and 24 h p.i. ($P < 0.001$) before reaching comparable mean titers at 48 h p.i. Despite the strain-specific differences observed between viruses isolated within each wave, Anhui/1/13, Taiwan/1/14, Taiwan/2/14, and BC/1/15 replicated most efficiently at 37°C in Calu-3 cells, with mean titers of $>10^8$ PFU/ml by 24 h p.i. Ultimately, all H7N9 viruses tested reached peak mean titers of $>10^7$ PFU/ml by 48 h p.i., demonstrating the efficient replication capability of H7N9 viruses from all three waves in this cell type. These results indicate that while H7N9 viruses isolated over three waves replicate to high titer in human bronchial epithelial cells, they nonetheless have not acquired the ability to replicate efficiently at the temperature of the upper respiratory tract, a characteristic shared by other avian influenza viruses (39).

pH fusion threshold of first-, second-, and third-wave H7N9 viruses. It has been shown recently that the H7N9 virus Anhui/1/13 has a relatively elevated pH threshold (pH 5.8) for fusion (as determined by a virus-mediated cell-cell fusion assay) compared to that of a seasonal human H3N2 virus (pH 5.4) (40). Here, we used a virus-induced hemolysis assay to evaluate the pH fusion of H7N9 viruses from all three waves of H7N9 infection. Consistent with previous findings, the first-wave Anhui/1/13 virus maintained greater than 30% of its maximal hemolysis ability at pH 5.8 (Fig. 4). All other first- and second-wave H7N9 viruses tested shared pH profiles for fusion similar to that of Anhui/1/13 virus, showing approximately 10% or more of their maximal fusion activities at a fusion threshold of pH 5.8 (Fig. 4 and data not shown). However, H7N9 viruses from the third wave (BC/1/15 and HK/56/15) showed a slightly reduced pH threshold for fusion compared to that of the first- and second-wave H7N9 viruses. The highest pH value for the fusion of third-wave H7N9 viruses was 5.6, at which 20% of maximal fusion activity was remaining, indicating that the pH threshold for third-wave viruses has lowered

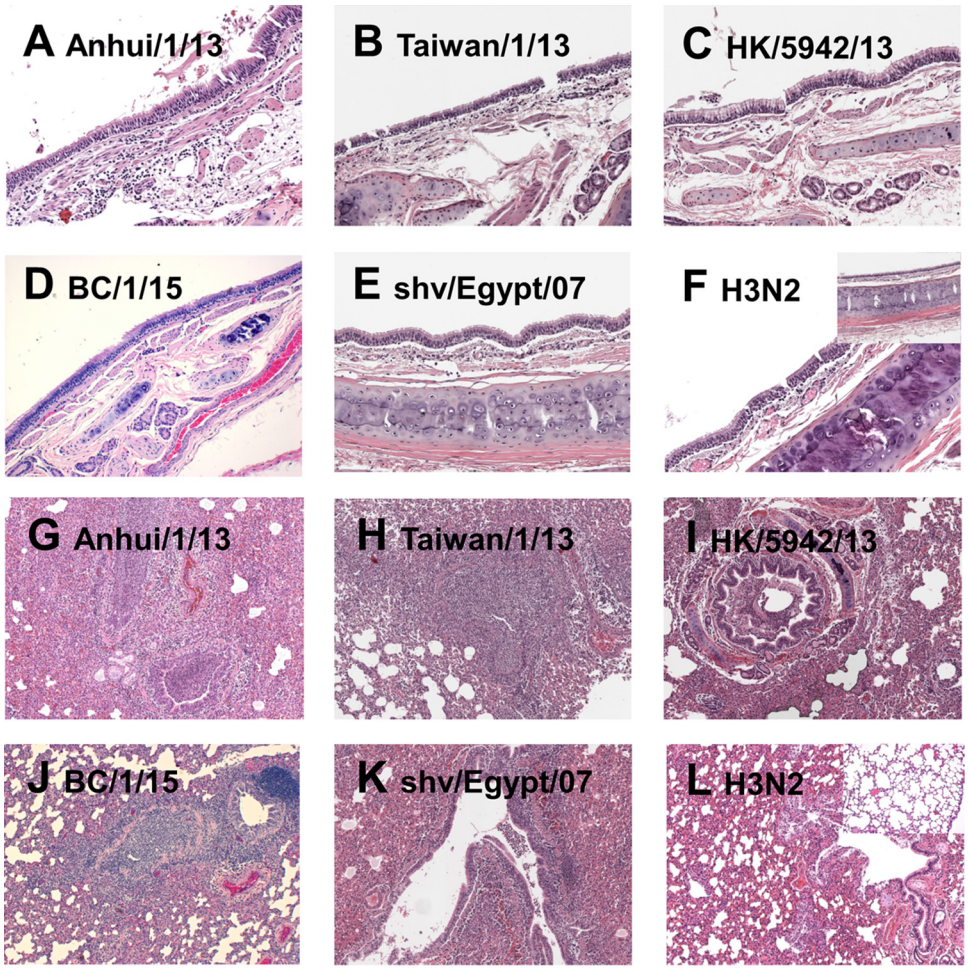

FIG 1 Representative images of histopathologic evaluation of ferrets infected with H7N9 and seasonal influenza viruses. Histopathologic evaluation using H&E staining shows a spectrum of inflammation in the respiratory tissue following infection with Anhui/1/13 (first wave) (A and G), Taiwan/1/13 (first wave) (B and H), HK/5942/13 (second wave) (C and I), BC/1/15 (third wave) (D and J), shv/Egypt/07 (precursor H7N9) (E and K), and A/Texas/50/12 (seasonal H3N2) (F and L) viruses. The airway tissues show minimal to moderate tracheobronchitis (A to F), while the lung tissues show mild to severe bronchopneumonia (G to L) in different groups of infected animals. The normal histology of ferret airway and lung tissues are shown as inserts in panels F and L. Original magnifications: airway tissue, ×200; lung tissue, ×50.

0.2 pH units, from pH 5.8 to pH 5.6. A reduction in the pH fusion threshold has been associated with avian influenza virus adaptation to mammalian hosts (41). Although highly transmissible influenza viruses typically resist fusion until reaching the acidic environment of the mammalian upper respiratory tract, the reduced pH fusion threshold of third-wave H7N9 viruses indicates a sign of adaptation toward humans, although further study is required due to the limited number of isolates from each wave tested here.

DISCUSSION

Since their initial detection in 2013, H7N9 viruses have exhibited a great expansion in both genetic diversity and geographical distribution (3, 25), representing an ongoing pandemic threat. Well-characterized mammalian H7N9 challenge models have been used widely to evaluate a broad range of parameters critical to public health, including the induction of inflammatory responses, the generation of effective vaccine approaches, and antiviral efficacy (42–44). However, these studies largely rely on the use of the very first human isolates from Anhui and Shanghai from spring 2013 (45). As H7N9 viruses continue to evolve (3), it is critical to determine if the pathogenesis and potential for transmission among mammals of currently circulating isolates remain similar to that of first-wave H7N9 viruses; while our study is not inclusive of all potential isolates from each wave, the representative viruses from each wave tested here provide a first attempt to analyze this virus subtype over three calendar years as it continues to cause human infection.

The generally comparable pathogenesis observed in both mouse and ferret models following intranasal inoculation with H7N9 viruses (Tables 3 and 5 and Fig. 2) is consistent with studies which have demonstrated that minor HA sequence variation between H7N9 viruses does not substantially influence virus attachment to the mammalian respiratory tract (46). Infectivity (MID_{50}), lethality (LD_{50}), and morbidity (weight loss) of H7N9 viruses in BALB/c mice were similar to those of highly pathogenic influenza viruses H5N1 and H7N7 in this model (13, 21), highlighting the capacity of this LPAI virus to cause severe mammalian disease. However, infection with the first-wave virus Taiwan/1/13, while main-

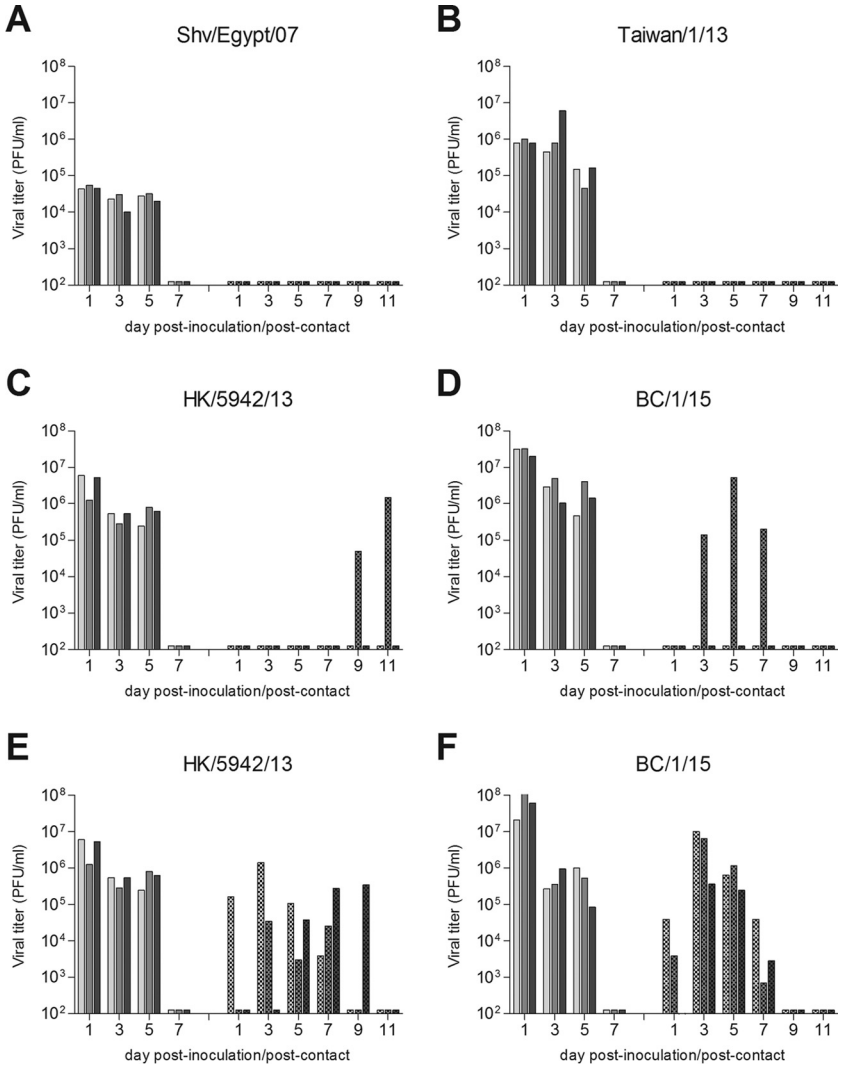

FIG 2 Transmissibility of H7N9 viruses in ferrets. Three ferrets were inoculated with 10^6 PFU of virus, and nasal washes were collected from each ferret on the indicated days p.i. (solid bars). A naive ferret was placed in a cage with perforated side walls adjacent to each inoculated ferret 24 h p.i. (A to D) or in the same cage as an inoculated ferret (E and F), and nasal washes were collected from each contact ferret on the indicated days p.c. (hatched bars) to assess virus transmission. Avian precursor (Shv/Egypt/07) (A), first-wave (Taiwan/1/13) (B), second-wave (HK/5942/13) (C and E), and third-wave (BC/1/15) (D and F) H7N9 influenza viruses were tested. The limit of virus detection was 100 PFU.

taining a virulent phenotype in mice, resulted in reduced morbidity and systemic spread of virus in ferrets compared with other H7N9 viruses associated with human infection (Table 5), in agreement with a prior study which also reported enhanced virulence in mice but not ferrets with this virus (47). While Taiwan/1/13 was the only virus in this study not bearing a lysine at position 226 in the HA, it is unlikely that this position alone contributed to the difference in mammalian virulence observed here, as the first-wave virus A/Shanghai/1/13, which exhibited virulence in mice and ferrets comparable to that of Anhui/1/13 virus, also lacks a lysine at this position (Table 2) (48). However, several NA variants of A/Taiwan/1/13 virus were detected in the original clinical isolate of this virus, with selected variants exhibiting reduced susceptibility to oseltamivir and attenuated phenotypes *in vitro* (47), suggesting that this isolate is unique compared with other first-wave isolates tested in previous studies.

H7N9 subtype viruses continue to distinguish themselves from other Eurasian and North American lineage H7 subtype viruses in their absence of an ocular tropism, which is typically observed with this subtype (49). Despite maintaining a strong tropism for the respiratory tract, the frequency of H7N9 virus detection in murine tissues following i.o. inoculation is comparable to that of other H7 subtype viruses, exceeding that observed with H5N1, seasonal, or 2009 H1N1 pandemic viruses (19, 50). These findings are in agreement with previous work in the ferret model, demonstrating the ability of a first-wave H7N9 virus to cause infection in ferrets following ocular exposure (51). While potential correlations between ocular tropism detected in the murine model and human susceptibility to influenza viruses following ocular exposure still are poorly understood, these data underscore the necessity of evaluating influenza viruses for the capacity to cause mammalian infection following alternate inoculation routes, even in the absence of a demonstrated tropism outside the respiratory tract.

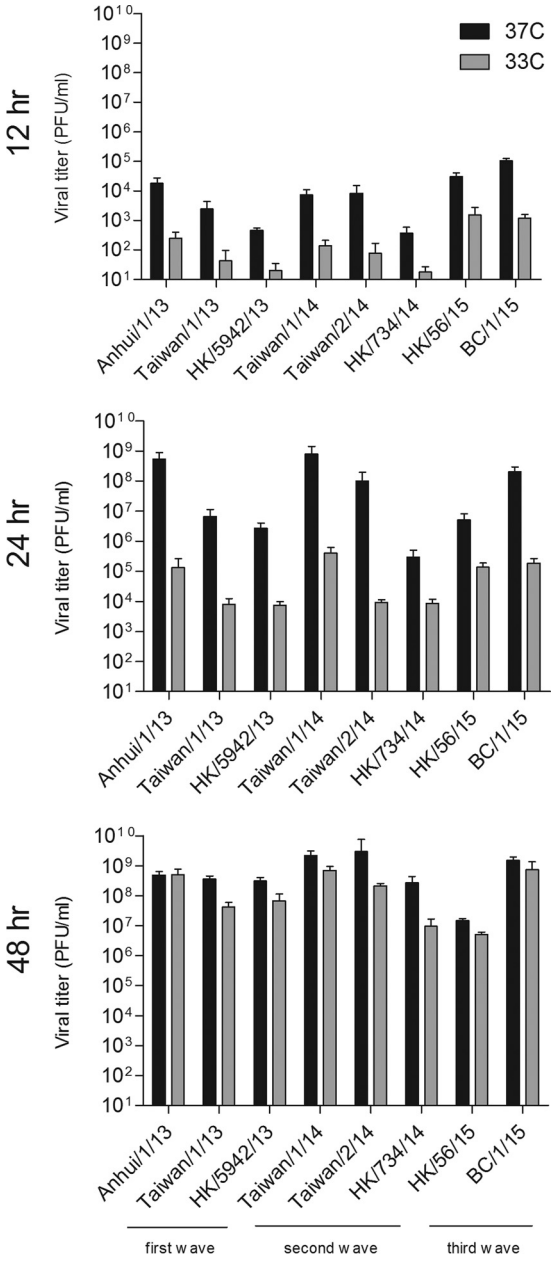

FIG 3 Replication kinetics of H7N9 viruses in polarized human airway epithelial cells. Calu-3 cells were inoculated with influenza viruses at an MOI of 0.01 and cultured at either 37°C or 33°C. Culture supernatants were collected at the times indicated, and virus titers were determined by plaque assay. Titer values represent the means plus standard deviations. The limit of detection was 10 PFU.

As observed in this study with shv/Egypt/07 virus, H7N9 viruses isolated from poultry from multiple waves have demonstrated reduced transmissibility in the ferret model compared with viruses isolated from humans (30, 31, 52). While this can be explained in part by sequence differences often present between poultry- and human-isolated viruses (notably at PB2 residue 627), further examination of how these viruses diverge will allow for a more precise understanding of the adaptation these viruses have undergone to cause human disease. Mutations in the polymerase genes highlighted in Table 2 may have increased in frequency as a result of selection during circulation in poultry, but they appear to have minimal impact on virus replication and transmission in mammals. The limited transmissibility by respiratory droplets of second- and third-wave H7N9 viruses in ferrets tested here aligns with other studies which have reported transmission primarily among cohoused animals (52). Importantly, the similar transmission profile of H7N9 viruses between first-wave isolates and viruses isolated during the second and third waves suggest that vaccine and antiviral candidates which limit the transmissibility of the commonly employed Anhui/1/13 virus would maintain a comparable efficacy against more recently detected H7N9 viruses (53).

A previous study suggested that a high viral load (and associated induction of host responses) following the infection of bronchial epithelial cells with first-wave H7N9 viruses results in decreased integrity of the epithelial monolayer, leading to the infection of underlying endothelial cells, which ultimately may contribute to the severe pneumonia associated with H7N9 virus infection in humans (54). The high capacity for H7N9 viruses to replicate in a human respiratory tract cell type shown in this study (Fig. 3) indicates that H7N9 viruses isolated during the second and third waves have maintained this property. The induction of host innate immune responses represents a critical component of the virulent phenotype of H5N1 viruses in mammals (55). Interestingly, first-wave H7N9 viruses have been shown to be poor inducers of proinflammatory cytokines and chemokines compared with other avian viruses (such as H5N1) associated with severe human respiratory disease (54, 56, 57). While not examined here, these data suggest that H7N9 viruses from 2013 to 2015 would function similarly to first-wave viruses in this regard.

The study of viral fusion pH has attracted increased attention due to recent evidence that this property influences virus stability, transmission, and host adaptation (58, 59). In general, human influenza viruses tend to fuse at relatively lower pH values in order to avoid being inactivated prior to reaching the acidic nasal passageways, whereas most avian influenza viruses preferentially fuse at higher pH values, leading to the potential advantage of more rapid fusion at early endosomes during viral entry (60). As demonstrated in the ferret transmission model, airborne-transmissible H5N1 viruses acquired not only receptor binding switching mutations but also mutations lowering fusion pH, emphasizing that having a reduced pH for fusion might be necessary for avian influenza virus to initiate airborne transmission in mammalian hosts (58, 59). The relatively high fusion threshold (pH 5.8) of novel H7N9 viruses shown in our study and by other investigators (34, 40) may partially account for its limited transmission in humans. Interestingly, both third-wave H7N9 viruses examined here possessed slightly shifted pH fusion thresholds (pH 5.6) compared with those of first- and second-wave viruses, which indicates improved viral survival prior to reaching the acidic upper airways. However, based on the available H7N9 virus sequences from different waves, we cannot identify any mutations in the HA, NA, or M genes that are shared by all H7N9 viruses from specific waves, suggesting that the substitutions modulating viral fusion pH are strain specific. Continued studies individually examining fusion thresholds of newly emerging H7N9 viruses, and the impact these findings may have on transmission potential, are warranted.

It is likely that H7N9 viruses will continue to cause human infection in China, leading to persistent reassortment, diversity,

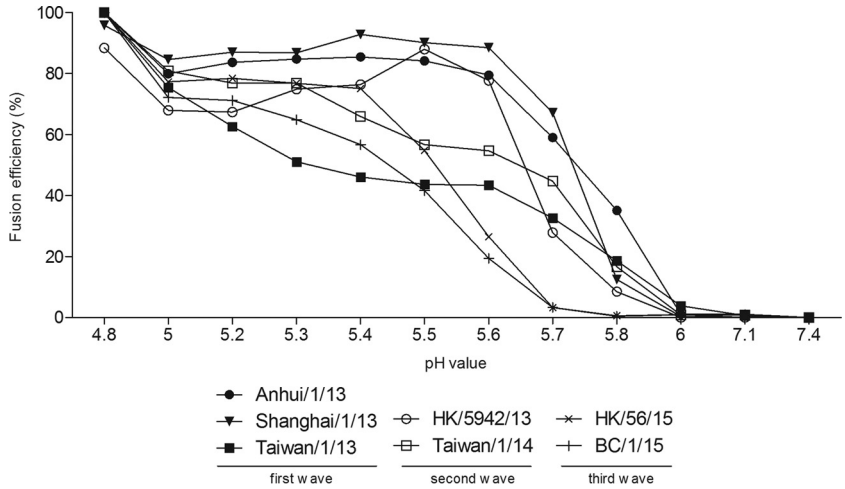

FIG 4 pH fusion threshold of H7N9 viruses. The hemolysis of turkey red blood cells following incubation with influenza virus or PBS mock-treated control at the indicated pH values was measured by optical density (OD) at 405 nm. The percentage of maximal hemolysis at each pH value was expressed as the average from three independent repeats. Solid shapes, first-wave H7N9 viruses; open shapes, second-wave H7N9 viruses; cross-hatch marks, third-wave H7N9 viruses.

and geographic spread. Our findings that viruses from the first three waves have maintained a pathogenic phenotype in mammals and upheld a robust transmissibility in the presence of direct contact with a capacity for transmission by respiratory droplets suggest that these viruses remain as immediate a threat to public health as when they were first detected in humans in 2013. However, given the phylogenetic diversity among H7N9 viruses and the relatively small number of viruses tested *in vivo* from each wave here, further studies with additional isolates from these waves are warranted to confirm the absence of strain-specific effects. As human infection with H7N9 viruses has reached a fourth wave of human cases, a greater understanding of how these viruses continue to evolve, and how this may influence mammalian pathogenicity and transmissibility, will support the continued generation of preventative measures required to limit and prevent the spread of H7N9 viruses to humans.

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