Genetically and Antigenically Divergent Influenza A(H9N2) Viruses Exhibit Differential Replication and Transmission Phenotypes in Mammalian Models

Jessica A. Belser,[a] Xiangjie Sun,[a] Nicole Brock,[a] Claudia Pappas,[a] Joanna A. Pulit-Penaloza,[a] Hui Zeng,[a] Yunho Jang,[a] Joyce Jones,[a] Paul J. Carney,[a] Jessie Chang,[a] Nguyen Van Long,[b] Nguyen Thi Diep,[b] Sharmi Thor,[a] Han Di,[a] Genyan Yang,[a] Peter W. Cook,[a] Hannah M. Creager,[a] Dayan Wang,[d] Jeffrey McFarland,[c] Pham Van Dong,[b] David E. Wentworth,[a] Terrence M. Tumpey,[a] John R. Barnes,[a] James Stevens,[a] C. Todd Davis,[a] Taronna R. Maines[a]

[a]Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA
[b]Department of Animal Health, Ministry of Agriculture and Rural Development of Vietnam, Hanoi, Vietnam
[c]Office of the U.S. Centers for Disease Control and Prevention in Vietnam, Hanoi, Vietnam
[d]National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China

ABSTRACT Low-pathogenicity avian influenza A(H9N2) viruses, enzootic in poultry populations in Asia, are associated with fewer confirmed human infections but higher rates of seropositivity compared to A(H5) or A(H7) subtype viruses. Cocirculation of A(H5) and A(H7) viruses leads to the generation of reassortant viruses bearing A(H9N2) internal genes with markers of mammalian adaptation, warranting continued surveillance in both avian and human populations. Here, we describe active surveillance efforts in live poultry markets in Vietnam in 2018 and compare representative viruses to G1 and Y280 lineage viruses that have infected humans. Receptor binding properties, pH thresholds for HA activation, *in vitro* replication in human respiratory tract cells, and *in vivo* mammalian pathogenicity and transmissibility were investigated. While A(H9N2) viruses from both poultry and humans exhibited features associated with mammalian adaptation, one human isolate from 2018, A/Anhui-Lujiang/39/2018, exhibited increased capacity for replication and transmission, demonstrating the pandemic potential of A(H9N2) viruses.

IMPORTANCE A(H9N2) influenza viruses are widespread in poultry in many parts of the world and for over 20 years have sporadically jumped species barriers to cause human infection. As these viruses continue to diversify genetically and antigenically, it is critical to closely monitor viruses responsible for human infections, to ascertain if A(H9N2) viruses are acquiring properties that make them better suited to infect and spread among humans. In this study, we describe an active poultry surveillance system established in Vietnam to identify the scope of influenza viruses present in live bird markets and the threat they pose to human health. Assessment of a recent A(H9N2) virus isolated from an individual in China in 2018 is also reported, and it was found to exhibit properties of adaptation to humans and, importantly, it shows similarities to strains isolated from the live bird markets of Vietnam.

KEYWORDS H9N2, influenza, surveillance studies, transmission

Widespread detection of low-pathogenicity avian influenza (LPAI) A(H9N2) viruses in gallinaceous poultry (i.e., domestic and game birds) has been reported over the past 20 years in Asia, the Middle East, and regions of Africa, with periodic detection in Europe and the Americas (1). There is extensive genetic diversity present among A(H9N2) viruses, with over a hundred distinct genotypes and several discrete lineages currently identified (2). Documented A(H9N2) human cases or evidence of human

A(H9N2) virus infection or exposure via serologic studies have been reported in over a dozen countries (1, 3), highlighting the capacity for this virus subtype to cause mild and/or asymptomatic infections in humans. To date, confirmed human cases have been limited to the G1 and Y280 lineages (also known as the BJ94 or G9 lineages based on the A/chicken/Beijing/1/1994 and A/chicken/Hong Kong/G9/1997 prototype viruses, respectively).

While human-to-human transmission of A(H9N2) viruses has not been reported, viruses from both lineages have caused human infection and display enhanced binding to human-like α2,6-linked sialic acid receptors (4, 5). Multiple influenza virus subtypes associated with human infection, notably A(H7N9) viruses, possess internal genes derived from A(H9N2) viruses (6, 7), indicating that reassortment of avian influenza viruses with A(H9N2) viruses in poultry may enhance their capacity to adapt to mammalian hosts. A(H9N2) influenza viruses also possess heterogeneity in their threshold pH for fusion, with select viruses exhibiting pH stability similar to human transmissible viruses (8). Moreover, enhanced transmissibility following mammalian adaptation has been observed using the ferret model (4, 9). Finally, the lack of immunity to A(H9N2) subtype viruses in the general human population, coupled with their increasing genetic and antigenic diversity, adds to their public health threat and pandemic potential and the need for prepandemic candidate vaccine virus (CVV) assessment and development (1, 3).

Live bird markets (LBMs) can facilitate the circulation and reassortment of avian influenza viruses and are an ideal interface for human exposure to infected poultry and emergence of novel viruses. Approximately 70% of persons with confirmed A(H9N2) virus infection have reported poultry exposure prior to infection (3). In Vietnam, LBMs are common throughout the country, connecting farms with distributors, and play a substantial role in the poultry industry. As such, these markets are often sentinel locations for the identification of endemic and emerging avian influenza viruses and serve as critical sites from which to conduct active surveillance. Collection of samples from infected birds across national surveillance networks in Vietnam results in the testing of thousands of avian influenza viruses each year, allowing for their genetic and antigenic analysis as well as additional virologic characterization required for virus risk assessment.

Here, we examined genetically and antigenically diverse poultry viruses from Vietnam and compared them to contemporary LPAI A(H9N2) viruses inclusive of both G1 and Y280 lineages isolated from recent human infections. Genetic, antigenic, and receptor binding site characterization identified further divergence of recently detected A(H9N2) viruses and the need for continued CVV development. We found that A(H9N2) viruses were capable of high-titer replication in human respiratory tract cells and throughout the respiratory tract of both mice and ferrets. Transmission was efficient between cohoused ferrets, while a recent virus isolated following a human infection in China was capable of robust transmission through the air.

RESULTS

LBM surveillance and HA gene diversity. Surveillance in LBMs in Vietnam from September 2017 to September 2018 detected 163 A(H9N2) positive oropharyngeal swabs and environmental samples. A(H9N2) viruses made up 36% of the total avian influenza viruses detected. Other subtypes identified included H2N3, H3N2, H3N8, H5N1, H5N6, H6N2, H6N6, H7N6, H10N3, and H11N9. Codon complete genomic sequencing of viral RNAs was successful for 55 new Y280-lineage sequences from Vietnam. Viruses clustered with previously described Y280 lineage viruses, including the Y280 CVV, A/Hong Kong/308/2014 (HK/308). Viruses from Vietnam exhibited extensive genetic diversity clustering in multiple small groups and two larger overarching lineages classified as the A Y280 and B Y280 lineages (Fig. 1A and B). The A Y280 lineage viruses were collected from both northern, central and southern Vietnamese provinces, while B Y280 viruses were collected almost exclusively from central and northern provinces (see Table S1 in the supplemental material). Some clusters from both lineages
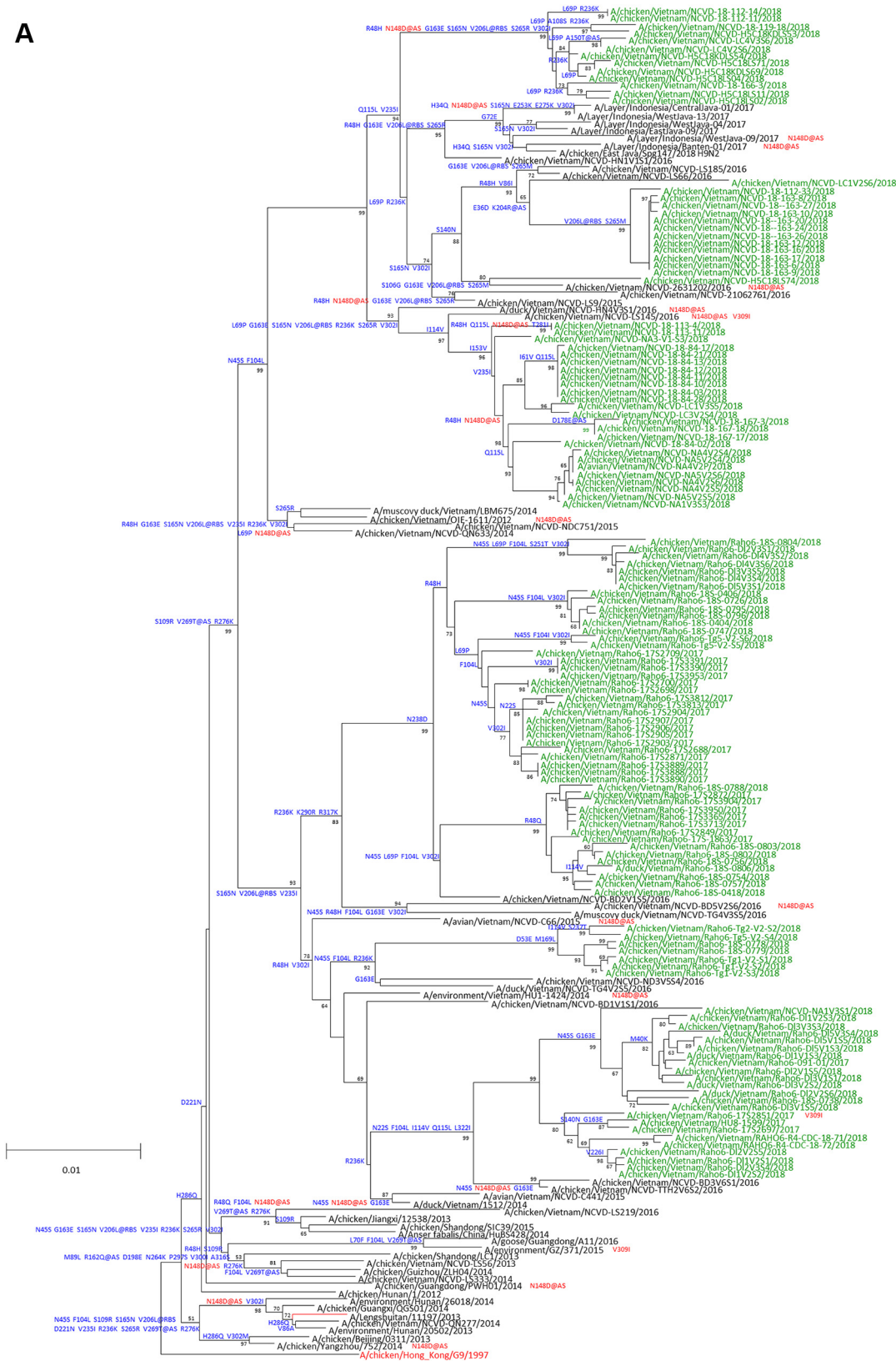
FIG 1 Evolution of A(H9N2) HA genes belonging to the Y280 lineages A and B. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. (A and B) The A Y280 (A) and B Y280 (B) viruses tested in this study are shown in green. Candidate vaccine viruses are shown in red. Amino acid differences relative to the closest candidate vaccine viruses are shown in blue on each tree branch. Mutations found in the genetic changes inventory are shown in red. Bold lettering indicates a mutation at a putative antigenic site.

FIG 1 (Continued)

contained previously identified A(H9N2) viruses from Vietnam and likely represented endemically circulating strains found in Vietnam since at least 2016. Other groups had no close ancestors to previous Vietnam viruses, suggesting recent emergence or introduction into Vietnam. One cluster of viruses in the A Y280 lineage was collected in northern provinces bordering China (i.e., Lang Son, Lao Cai, and Quang Nin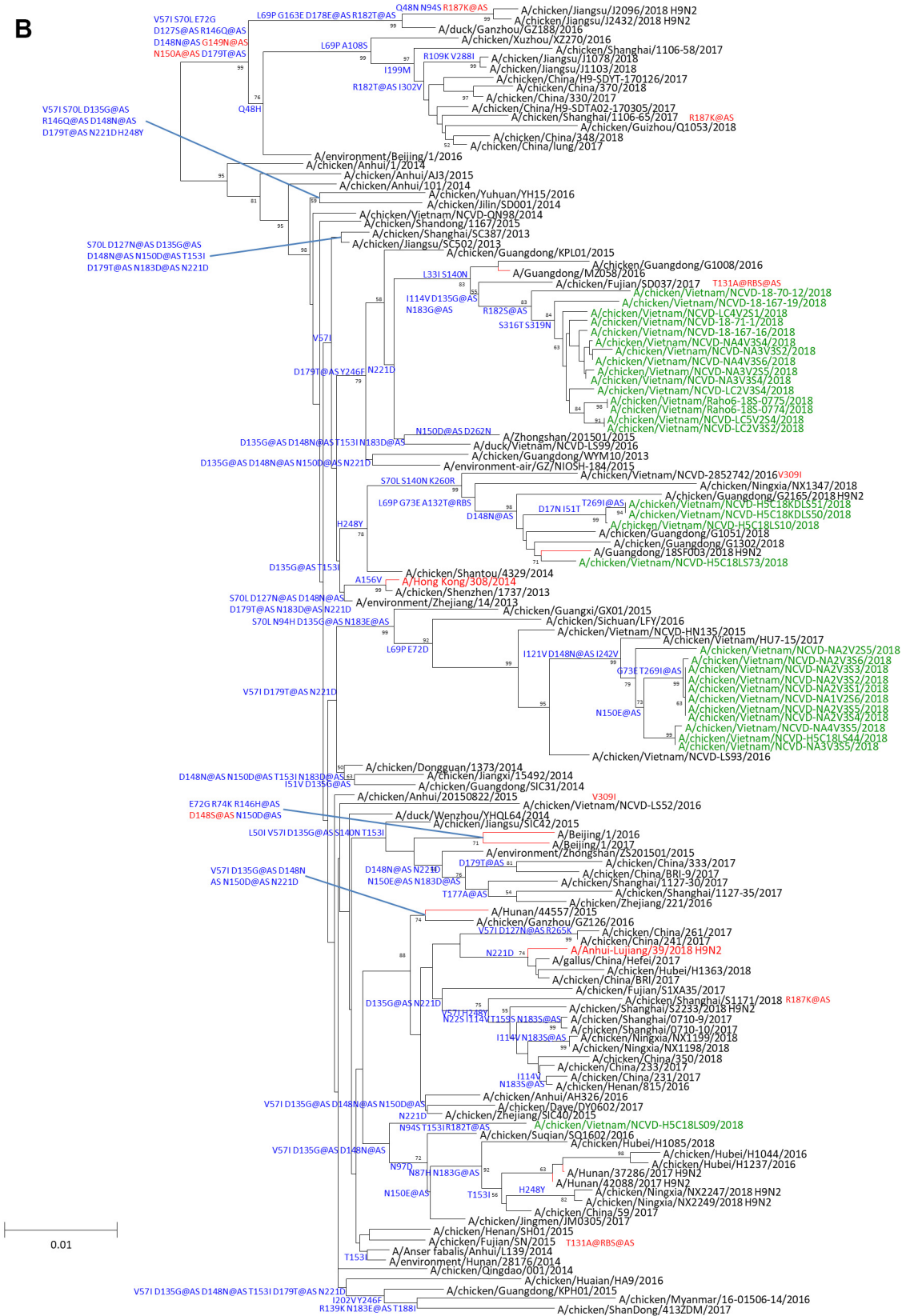h provinces) (Fig. 1A). The nearest ancestors were viruses detected in China in 2017 and 2018, suggesting introduction via shorter-distance poultry trade across porous borders. Three clusters of viruses belonging to the B Y280 lineage were identified with no known ancestors previously detected in Vietnam (Fig. 1B). One group contained viruses from northern border provinces with close phylogenetic proximity to viruses collected in Guangdong and Fujian provinces, China. Another group in the B Y280 lineage (collected in the northern border province of Lang Son) was closely related to viruses from Myanmar detected in 2018, suggesting more distant but rapid dispersal of these viruses in southeast Asia (Fig. 1B). Finally, a third cluster, also collected from Lang Son province, was related to poultry viruses from Guangdong province that caused a human infection in the same province in 2018. One A(H9N2)-positive environmental sample from Vinh Long province (Mekong Delta region of Vietnam) encoded HA viral RNA that belonged to the Eurasian/American wild bird lineage and was genetically distinct from all recommended A(H9N2) CVVs (data not shown).

Molecular analysis of LPAI A(H9N2) viruses. Representative H9N2 viruses isolated from LBM surveillance in Vietnam (inclusive of multiple lineages and genetic diversity) were chosen for subsequent analyses, contextualized with viruses isolated from recent human cases from both G1 and Y280 lineages. Viruses isolated from humans and tested in this study were collected through routine diagnostic detections of A(H9N2) infections reported by national authorities in Bangladesh, Hong Kong, and China. The majority of cases were detected in children with mild illness. Most cases reported direct contact with poultry prior to illness onset, and all cases recovered from infection with no evidence of further human-to-human transmission (10–12).

A Y280 viruses from Vietnam were genetically close to the A/chicken/Hong Kong/ G9/97 CVV but possessed 23 to 26 amino acid differences in HA1 (Fig. 1A). Conserved amino acid differences were found in antigenic sites B (N148D), C (V269T), D (R162Q and Q217M) and E (R48H), but no differences resulted in changes in glycosylation status. Compared to the currently recommended CVV, A/Anhui-Lujiang/39/2018 (A-L/ 39, a human isolate from 2018), the B Y280 Vietnam viruses had 9 to 17 amino acid changes in the HA1. Three conserved amino acid substitutions in the HA1 of these viruses were in putative antigenic sites A (D135G) and B (D179T and N183G/E) but also did not result in glycosylation changes. Compared to HK/308 virus, B Y280 Vietnam viruses had between 7 to 23 amino acid differences in the HA1 protein (Fig. 1B). Despite the phylogenetic diversity of A and B Y280 HA genes, the viruses had similar amino acid residues at positions associated with receptor specificity and markers of mammalian adaptation potential and shared the same receptor specificity residues (i.e., Leu-Met-Gly at positions 226 to 228 by H3 numbering), indicating potential for dual-receptor specificity. This motif was shared by the Y280 human viruses tested in this study, while the G1 lineage human viruses had various combinations (Table 1). A Y280 viruses had predominately Ala at position 190, while B Y280 avian and human viruses had Thr, suggesting a potential difference in receptor specificity determined by the 190 helical domain. Both avian and human Y280 viruses had an Asn at position 183 associated with altered receptor binding specificity (Table 1).

The neuraminidase (NA) genes clustered with topologies similar to the HA genes (data not shown). At the amino acid level, only a small number of B Y280 viruses ($n = 26$) had a deletion of three amino acids (NA 62 to 64), indicative of adaptation to terrestrial poultry. This deletion was found in both of the Y280 human viruses characterized (Table 1). The presence of 294R among all H9N2 viruses evaluated here predicts sensitivity to the NA inhibitor oseltamivir. With the exception of A/Hong Kong/33982/ 2009, none of the PB2 proteins of the viruses characterized had 627K or 701N but

TABLE 1 Key molecular features and threshold pH of fusion of A(H9N2) viruses used in this study

| Virus name | Abbreviation | Lineage | HA[a] | | | | | NA | PB2 | | PB1 | PA | pH of fusion |
			183 (173)	190 (180)	226 (216)	227 (217)	228 (218)	del62-64	627	701	368	185	
A/Hong Kong/1073/1999	HK/1073	G1	H	E	L	Q	G	no	E	D	I	K	5.8
A/Hong Kong/33982/2009	HK/33982	G1	H	D	Q	Q	G	no	E	N	I	K	5.5–5.8
A/Bangladesh/0994/2011	Bang/0994	G1	H	A	L	I	G	no	E	D	I	K	5.6–5.7
A/Hong Kong/308/2014	HK/308	B Y280	N	T	L	M	G	yes	E	D	V	R	5.4–5.5
A/Anhui-Lujiang/39/2018	A-L/39	B Y280	N	T	L	M	G	yes	V	D	V	R	5.4
A/ck/Vietnam/NCVD-18-70-12/2018	ck/VN/70	B Y280	N	T	L	M	G	no	E	D	V	R	5.5
A/ck/Vietnam/NCVD-NA4V3S5/2018	ck/VN/NA4	B Y280	N	T	L	M	G	yes	E	D	V	R	5.3–5.4
A/ck/Vietnam/NCVD-18-119-18/2018	ck/VN/119	A Y280	N	A	L	M	G	no	E	D	V	R	5.3–5.4
A/ck/Vietnam/NCVD-18-167-18/2018	ck/VN/167	A Y280	N	A	L	M	G	no	E	D	V	R	5.4–5.5

[a]For HA, H3 numbering is shown, with H9 numbering provided in parentheses.

instead possessed avian-consensus amino acids 627E/V and 701D. Additionally, while the G1 lineage human viruses tested contained an Ile at position 368 of the PB1 protein, the majority of Y280 viruses, including the human viruses, had a Val at this position. Finally, the PA proteins differed between the G1 and Y280 lineage viruses with the later lineage possessing Arg at position 185 (Table 1). GiRaF reassortment analysis did not identify reassortment within gene segments of the A(H9N2) viruses sequenced for this study (data not shown). In addition, there were no reassortment events confirmed among the A(H9N2) human-origin viruses analyzed in this study.

Antigenic characteristics of LPAI A(H9N2) viruses. Hemagglutination inhibition (HI) testing of 14 recent A(H9N2) Y280 lineage viruses from Vietnam was performed (Table 2). Ferret antiserum raised to the A/chicken/Hong Kong/G9/1997-like CVV (IBCDC-RG2) inhibited hemagglutination of seven A Y280 viruses at titers that were equivalent or within 2-fold of the homologous virus titer. However, this antiserum had no cross-reactive titers with B Y280 viruses. In contrast, ferret antisera raised to wild-type A-L/39 virus had heterologous titers against six B Y280 Vietnam viruses that were within 4-fold of the homologous virus titer but significantly reduced heterologous

TABLE 2 Hemagglutination inhibition assay using representative A(H9N2) viruses

Antigen[a]	Lineage	G1 Bang/0994	G1 RG-31	A Y280 RG-2	B Y280 HK/308	A-L/39	Date collected[c]
Reference antigens							
A/Bangladesh/0994/2011	G1	**_5120_**[b]	2,560	40	10	40	2/26/2011
A/Bangladesh/0994/2011 IDCDC RG-31	G1	5,120	**_2560_**	80	10	40	NA
A/chicken/Hong Kong/G9/1997 IBCDC RG-2	A Y280	640	20	**_320_**	10	<10	NA
A/Hong Kong/308/2014	B Y280	40	10	<10	**_1280_**	640	12/28/2013
A/Anhui-Lujiang/39/2018	B Y280	80	<10	<10	320	**_5120_**	1/2/2018
Test Antigens							
A/chicken/Vietnam/NCVD-18-119-18/2018	A Y280	640	80	160	80	160	3/11/2018
A/chicken/Vietnam/NCVD-H5C18LS02/2018	A Y280	640	80	160	80	160	2/11/2018
A/chicken/Vietnam/NCVD-H5C18KDLS54/2018	A Y280	1280	160	320	160	320	3/15/2018
A/chicken/Vietnam/NCVD-18–163-16/2018	A Y280	640	80	160	80	160	6/4/2018
A/chicken/Vietnam/NCVD-18-84-11/2018	A Y280	1280	80	160	80	160	8/2/2018
A/chicken/Vietnam/NCVD-18-113-11/2018	A Y280	640	80	160	80	160	3/11/2018
A/chicken/Vietnam/NCVD-18-167-18/2018	A Y280	640	80	320	160	320	9/4/2018
A/chicken/Vietnam/NCVD-NA4V2S6/2018	A Y280	640	80	160	80	160	3/7/2018
A/chicken/Vietnam/Raho6-18S-0775/2018	B Y280	80	<10	<10	40	1280	4/5/2018
A/chicken/Vietnam/NCVD-LC5V2S4/2018	B Y280	80	<10	<10	320	5120	8/3/2018
A/chicken/Vietnam/NCVD-18-70-12/2018	B Y280	80	<10	<10	40	1280	3/4/2018
A/chicken/Vietnam/NCVD-H5C18KDLS50/2018	B Y280	40	<10	<10	320	2560	3/15/2018
A/chicken/Vietnam/NCVD-NA4V3S5/2018	B Y280	40	<10	<10	640	1280	5/4/2018
A/chicken/Vietnam/NCVD-H5C18LS09/2018	B Y280	20	<10	<10	160	2560	2/11/2018

[a]A/Bangladesh/0994/2011 (Bang/0994); A/Bangladesh/0994/2011 IDCDC RG-31 (RG-31); A/chicken/Hong Kong/G9/1997 IBCDC RG-2 (RG-2); A/Hong Kong/308/2014 (HK/308); A/Anhui-Lujiang/39/2018 (A-L/39).
[b]Hemagglutination inhibition titers represent the reciprocal of the titer that inhibited complete hemagglutination with a starting dilution of 1:10. Homologous hemagglutination titers are indicated in bold and underlined.
[c]NA, not applicable.

TABLE 3 Hemagglutination of A(H9N2) viruses using resialylated red blood cells

Virus	Subtype	HA titer[a]			
		TRBC	α2-6 SA TRBC	α2-3 SA TRBC	Desialylated TRBC
HK/1073	A(H9N2)	2,048	4,096	8,192	<2
HK/33982	A(H9N2)	4,096	4,096	8,192	<2
Bang/0994	A(H9N2)	2,048	4,096	4,096	<2
HK/308	A(H9N2)	4,096	8,192	4,096	<2
A-L/39	A(H9N2)	4,096	8,192	4,096	<2
ck/VN/70	A(H9N2)	1,024	2,048	1,024	<2
ck/VN/NA4	A(H9N2)	2,048	2,048	2,048	<2
ck/VN/119	A(H9N2)	1,024	1,024	<2	<2
ck/VN/167	A(H9N2)	2,048	2,048	<2	<2
Shanghai/1	A(H7N9)	2,048	4,096	4,096	<2
Cal/7	A(H1N1)	32	64	<2	<2
Switz/13	A(H3N2)	1,024	1,024	512	<2

[a]HA titers were measured with untreated turkey red blood cells (TRBC), *Vibrio cholerae*-treated TRBCs (desialylated), or α2,6 or α2,3 resialylated TRBCs. HA titer is representative of duplicate experiments and is the reciprocal of the highest dilution that results in agglutination of TRBC.

titers (16- to 32-fold) to the A Y280 viruses (Table 2). In addition, ferret antisera raised to the Y280 wild-type virus, A/Hong Kong/308/2014, had heterologous titers that were, on average, 8-fold or greater reduced with both A and B Y280 Vietnam viruses compared to the homologous virus titer, indicating recent antigenic drift among viruses from both Y280 lineages.

A(H9N2) viruses exhibit a range of pH thresholds for HA activation. An increasing body of evidence has supported the requirement for an acid-stable hemagglutinin for efficient airborne transmission of influenza viruses in mammalian models, as this allows survival of the virus in acidic milieu of the upper airways (13). Previous studies have indicated that most transmissible human influenza viruses have a fusion threshold pH of <5.5, while most A(H5) and A(H7) subtype avian influenza viruses generally fuse at a pH of >5.5 (14). Accordingly, we examined the ability of A(H9N2) viruses to induce syncytium formation in Vero cells upon exposure to low-pH fusion buffers (15). We found that the fusion threshold pH, as defined as the highest pH value to induce at least 50% or more syncytium, was at or above 5.5 (range of 5.5 to 5.8; Table 1) for all G1 lineage viruses tested in this study. In contrast, the fusion thresholds for all Y280 lineage viruses tested were equal to or below pH 5.5 (range of 5.3 to 5.5; Table 1), including viruses isolated from LBMs in Vietnam. The lower threshold pH for fusion exhibited by these A(H9N2) viruses is characteristic of the acid stability displayed by human seasonal influenza viruses.

Receptor binding properties of A(H9N2) viruses. Human influenza viruses typically display a binding preference for α2,6-linked sialic acid receptors, while avian influenza viruses predominately bind to α2,3-linked sialic acids. Enhanced binding to human-like α2,6-linked sialic acids has been reported previously among human and avian A(H9N2) viruses (4, 5). To investigate whether recently isolated A(H9N2) viruses have maintained this capacity, we first employed a hemagglutination assay using differentially sialylated turkey red blood cells (RBCs). Comparable to the first-wave LPAI A(H7N9) virus, A/Shanghai/1/2013 (Shanghai/1), all G1 and B Y280 viruses bound to turkey RBCs sialylated with either α2,3- or α2,6-linked sialosides (Table 3), with all HA titers within a 2-fold difference. Interestingly, the two A Y280 A(H9N2) viruses bound only to α2,6-linked sialosides in this assay. These results support the notion that contemporary A(H9N2) viruses possess an ability to bind to α2,6-linked sialic acids, though strain-specific differences exist.

Next, a panel of recently isolated A(H9N2) viruses were subjected to glycan microarray analysis (Fig. 2) (16). In contrast to the RBC assay, which employs live virus and species-specific avian erythrocytes, glycan microarrays capture a complex profile of natural glycans from different species and tissue types. The G1 lineage virus HK/33982 bound well to α2,6 glycans, while Bang/0994 bound to a single α2,3/α2,6 di-
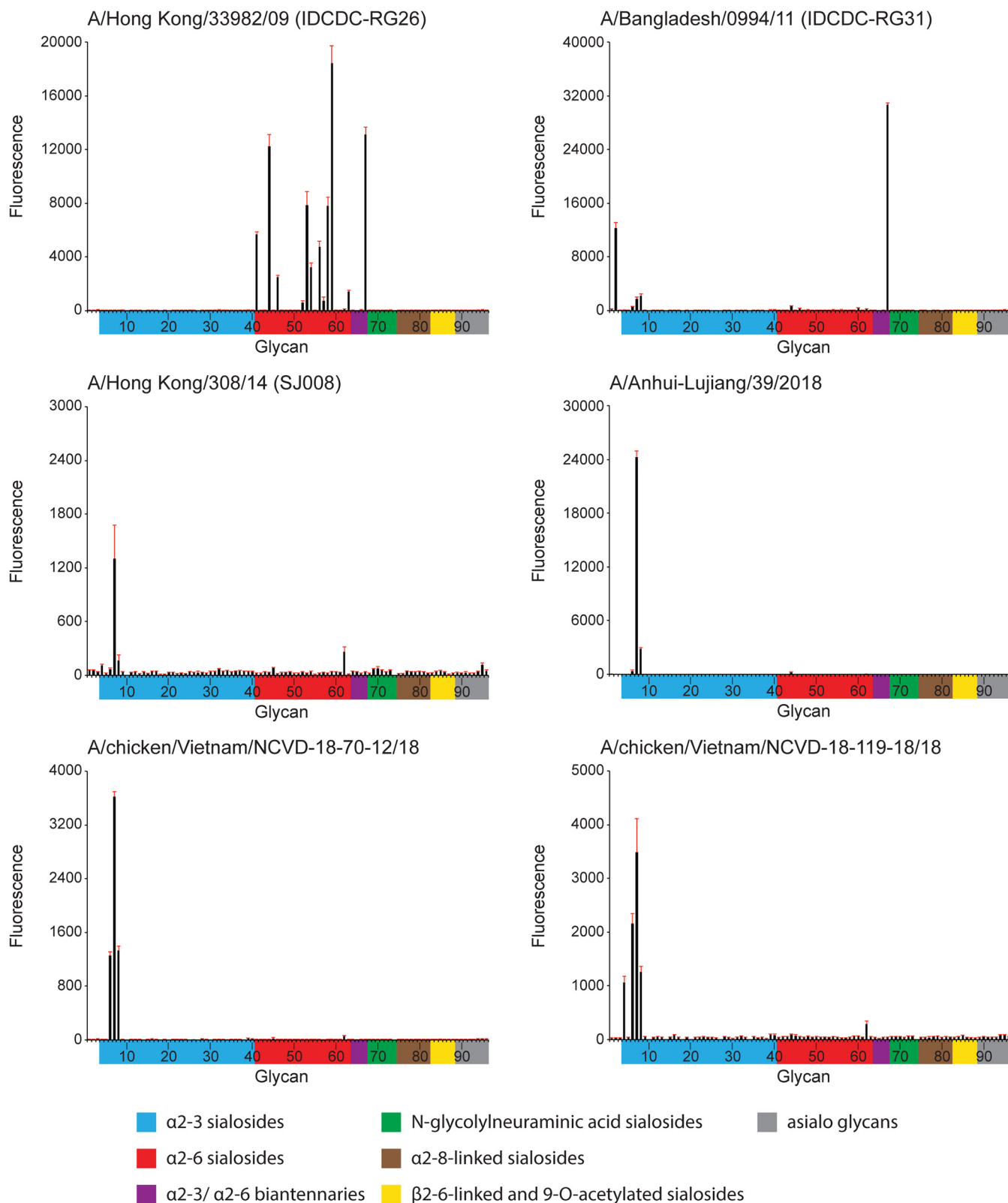
FIG 2 Glycan microarray analysis of representative A(H9N2) influenza viruses. Colored bars represent glycans that contain α-2,3 sialic acid (SA) (blue), α-2,6 SA (red), α-2,3/α-2,6 mixed SA (purple), N-glycolyl SA (green), α-2,8 SA (brown), β-2,6 and 9-O-acetyl SA (yellow), and non-SA (gray). Error bars reflect the standard error (SE) in the signal for 6 independent replicates on the array. Antisera for glycan array analysis were raised against homologous virus, with the exception of ck/VN/119 and ck/VN/70, for which antisera were raised against A-L/39 virus.

sialoside (glycan number 67). In contrast, A and B Y280 lineage viruses revealed only limited binding to α2,3 sulfated sialosides (glycan numbers 6 to 8). Glycan microarray binding was also performed using a recombinant A-L/39 virus and avian Viet04 and seasonal Switz/13 HAs as controls; data were consistent with the A-L/39 virus results (Fig. 3).

A(H9N2) virus replication in human and ferret respiratory tract cells. Previous studies have identified that A(H9N2) influenza viruses are capable of high-titer replication in human cultured respiratory tract cells and in *ex vivo* respiratory tract tissue samples (17, 18). However, recently isolated B Y280 viruses from human infections have not been extensively studied. To assess the capacity for contemporary A(H9N2) viruses from humans or chickens to replicate in a representative human respiratory tract cell line, Calu-3 cell cultures were inoculated with a panel of influenza A viruses and cultured at 37°C or 33°C, temperatures which emulate those of the human lower and upper respiratory tracts, respectively (Fig. 4).

All A(H9N2) viruses tested reached mean titers of $>10^6$ PFU/ml by 24 h postinfection (p.i.) at 37°C (Fig. 4A), demonstrating a high capacity for replication in this cell type, comparable to LPAI A(H7N9) viruses (represented here by the first-wave Shanghai/1 virus). Notably, A-L/39 virus replicated to significantly higher titer at 37°C than all other viruses examined at 24 h p.i. ($P < 0.05$). All A(H9N2) viruses reached mean peak titers by 48 h p.i. of $>10^8$ PFU/ml, with the exception of ck/VN/119 virus. In contrast, mean peak titers of the human 2009 pandemic A(H1N1) virus, A/California/7/2009 (Cal/7), were $<10^6$ PFU/ml at 48 h p.i., and the seasonal A(H3N2) virus, A/Switzerland/9715293/2013 (Switz/13), did not reach mean peak titers of $>10^8$ PFU/ml until 72 h p.i.

Avian influenza viruses typically replicate with reduced and delayed kinetics when incubated at 33°C, in contrast to human influenza viruses, which are often better suited for replication at the lower temperatures of the human upper airways (Fig. 4B) (19). In support of this, viral titers from cells infected by human A(H1N1) or A(H3N2) viruses cultured at 33°C were reduced by <1 log at 24 h p.i. compared with cells cultured at 37°C, while all A(H9N2) and A(H7N9) viruses tested exhibited titer reductions between 1.4 and 3.1 logs at this time point (Fig. 4C). Accordingly, culturing inoculated Calu-3 cells at 33°C resulted in delays in reaching mean peak titers for all viruses examined from 48 to 72 h (with two exceptions, A-L/39 and ck/VN/119). However, mean peak titers were within a log of one another for all A(H9N2) viruses tested at both temperatures by 72 h p.i. Taken together, all A(H9N2) viruses replicated efficiently in human bronchial epithelial cells, with the most recent A(H9N2) virus human isolate, A-L/39 virus, reaching the highest titers ($>10^9$ PFU/ml) at both culture temperatures.

To evaluate the capacity of A(H9N2) viruses associated with human infection to replicate in the respiratory epithelia of another mammalian species, we inoculated cultures of differentiated primary ferret nasal epithelial cells (FNEC) or tracheal epithelial cells (FTEC) with either A-L/39 or HK/1073 virus (Fig. 5). In agreement with data from Calu-3 cells, A-L/39 virus replicated to significantly higher titers ($P < 0.01$) in either FNEC or FTEC cultured at 37°C compared with HK/1073 virus. Comparable virus replication in primary FNEC cultures was detected at 37°C or 33°C. Collectively, these data support a robust capacity of A(H9N2) viruses in general, and A-L/39 virus in particular, to replicate to high titer in the mammalian upper respiratory tract.

Mammalian pathogenicity of H9N2 viruses. Infection with A(H9N2) influenza viruses has been reported in numerous mammalian species (1); these viruses are typically associated with mild to moderate disease in laboratory mammalian models, with occasional exceptions (4, 18). Selected human and avian A(H9N2) influenza viruses were examined in the BALB/c mouse model to determine their capacity for efficient virus replication *in vivo*. All A(H9N2) viruses isolated from humans and tested here (inclusive of both G1 and B Y280 lineages) were capable of infection and replication in this species (Table 4). However, only HK/1073 virus infection caused moderate morbidity (17% weight loss) following high-dose (10^6 50% egg infective does [EID_{50}])
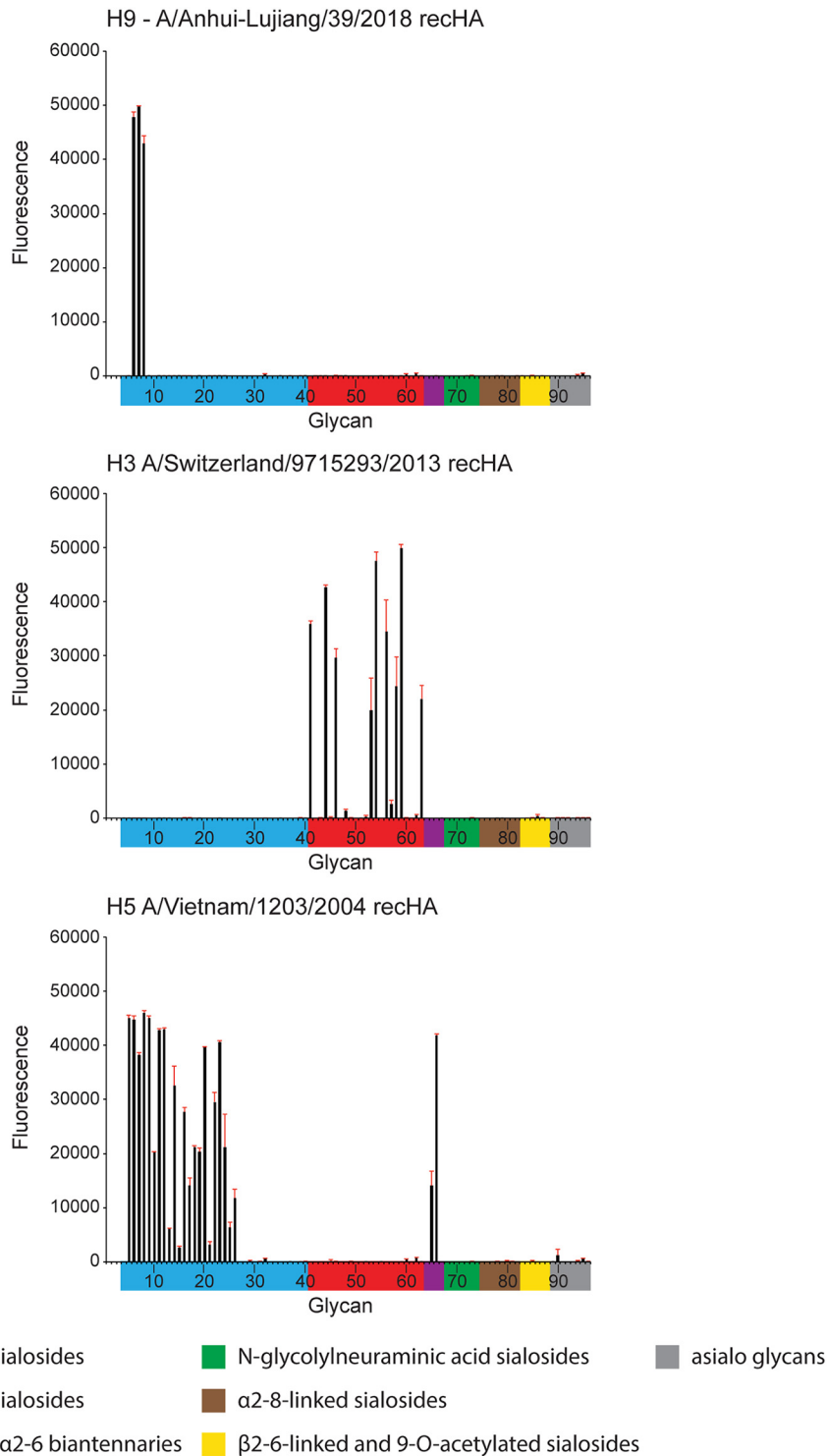
FIG 3 Glycan microarray analysis of recombinant HA controls. A-L/39, avian Viet04, and seasonal Switz/13 virus HAs were analyzed. Colored bars represent glycans that contain α-2,3 sialic acid (SA) (blue), α-2,6 SA (red), α-2,3/α-2,6 mixed SA (purple), N-glycolyl SA (green), α-2,8 SA (brown), β-2,6 and 9-O-acetyl SA (yellow), and non-SA (gray). Error bars reflect SE in the signal for 6 independent replicates on the array.

inoculation, and it was the only virus tested that was capable of productive replication in the lungs of mice after delivery of a lower dose (10^3 EID$_{50}$). Interestingly, mean viral titers in the nose remained elevated above 10^3 EID$_{50}$ through day 6 p.i. for all A(H9N2) human isolates examined. In contrast, the two chicken A(H9N2) viruses isolated in 2018
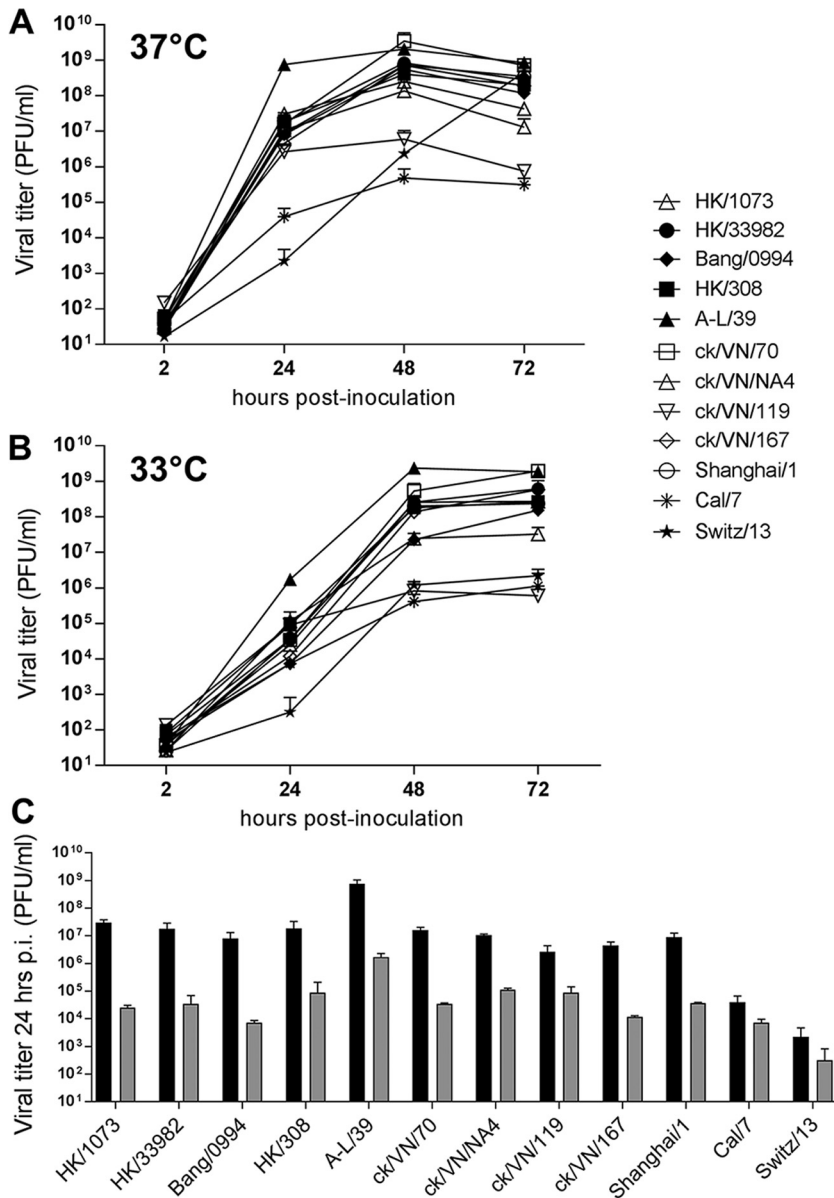
FIG 4 Replication of A(H9N2) viruses in Calu-3 cells. Human bronchial epithelial (Calu-3) cells were grown on transwell inserts and infected apically in triplicate at an MOI of 0.01 with the indicated A(H9N2), A(H7N9) (Shanghai/1), 2009 pandemic A(H1N1) (Cal/7), or seasonal A(H3N2) (Switz/13) viruses. (A and B) Cells were incubated at 37°C (A) or 33°C (B). Culture supernatants were sampled at the indicated times p.i. and titrated in MDCK cells by standard plaque assay. (C) Comparison of infectious titers collected at 24 h p.i. from 37°C (black bars) and 33°C (gray bars) cultures is shown. The limit of virus detection was 10 PFU/ml.

did not cause weight loss, were detected in fewer mice on day 3 p.i., and were cleared in all mice by day 6 p.i.

We next examined the capacity for selected A(H9N2) human isolates to cause disease in the ferret model, as the presentation of clinical signs of infection in ferrets is similar to humans, and humans and ferrets possess a high degree of similarity in respiratory tract tissues as well (20). Infection by either G1 (HK1073) or B Y280 (HK308 and A-L/39) lineage A(H9N2) viruses caused mild to moderate weight loss and transient fever in inoculated animals (Table 5); all ferrets survived the infection. Virus was detected at high titer in nasal wash (NW) specimens from all inoculated animals ($\geq 10^{6.8}$ EID_{50}/ml). Interestingly, the most recently isolated virus (A-L/39) caused the highest
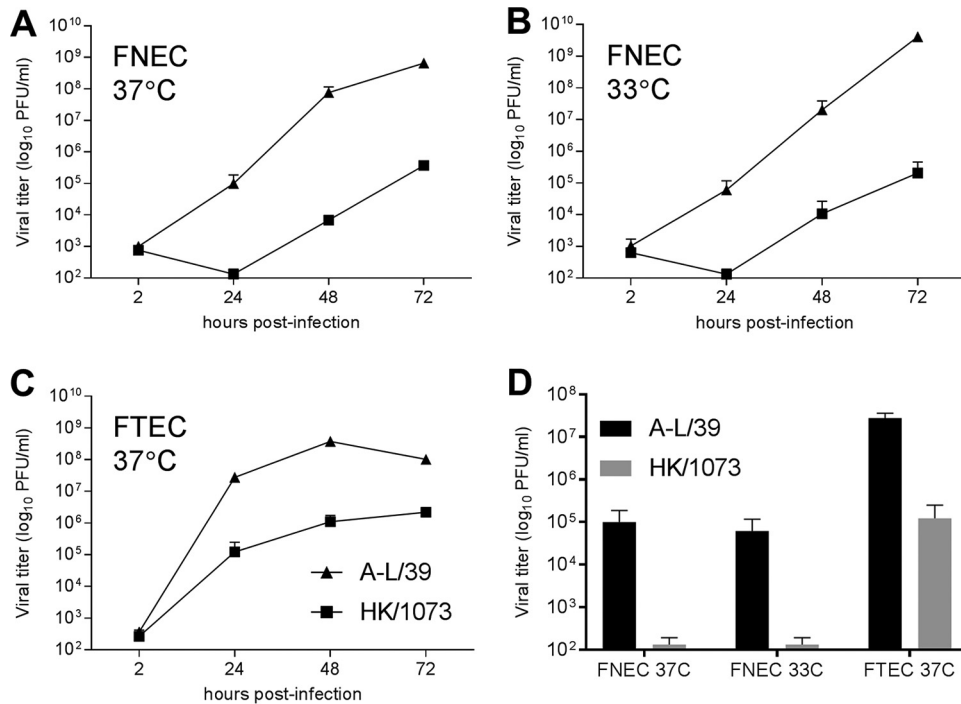
FIG 5 Replication of A(H9N2) viruses in primary ferret cell cultures. (A to C) Ferret nasal epithelial cells (FNEC) (A and B) or ferret tracheal epithelial cells (FTEC) (C) were isolated independently from three naive ferrets, grown on transwell inserts, and inoculated apically at an MOI of 0.01 with either A-L/39 or HK/1073 A(H9N2) virus. Cells were incubated at 37°C or 33°C as indicated. At each indicated time point, 200 μl of medium was added apically and collected after 10 min; all samples were titrated in MDCK cells by standard plaque assay. (D) Comparison of infectious titers collected at 24 h p.i. from all cultures is shown. The limit of virus detection was 10 PFU.

mean maximum weight loss (11.2%), highest mean peak titers in NW samples ($10^{8.2}$ EID_{50}/ml), and the highest frequency of nasal discharge/sneezing in inoculated ferrets.

Spread of A(H9N2) virus throughout the ferret respiratory tract and to extrapulmonary tissues was evaluated in ferrets 3 days after they received a 10^6 EID_{50} intranasal dose of virus (Fig. 6). All tested A(H9N2) viruses were detected throughout the ferret respiratory tract. A-L/39 virus displayed exceptional replicative ability and was the only virus detected at titers of $>10^5$ EID_{50}/g (or ml) in all of the respiratory tract tissues examined. Virus detection in the olfactory bulb and brain was sporadic, potentially influenced by high titers of virus in the nasal passages (21). A-L/39 virus was detected in both rectal swab (RS) specimens (peak titers of $10^{1.8}$ to $10^{2.3}$ EID_{50}/ml up to 5 days p.i.) and intestinal samples, while HK/308 and HK/1073 viruses were detected in RS (peak titers of $10^{2.0}$ to $10^{4.5}$ EID_{50}/ml) but not in intestinal tissues. Infectious virus was not detected in the liver, spleen, kidneys, or blood of any ferret examined (data not shown).

Multiple subtypes of human and avian influenza viruses have demonstrated the capacity to use ocular exposure as an entry route or have been associated with conjunctivitis or other ocular complications following infection (22). While A(H9N2) viruses have not been previously linked with ocular disease in humans, multiple human ocular cell types have been shown to support virus replication with this subtype (23). All ferrets inoculated with A(H9N2) virus in this study had infectious virus detected in conjunctival wash (CW) specimens up to 5 days p.i., with peak titers of $10^{3.3}$ to $10^{4.5}$ EID_{50}/ml (A-L/39 virus), $10^{1.0}$ to $10^{3.3}$ EID_{50}/ml (HK/308 virus), and $10^{2.5}$ to $10^{3.5}$ EID_{50}/ml (HK/1073 virus). Furthermore, infectious virus was detected in both the eye and surrounding conjunctiva on day 3 p.i. for all A(H9N2) viruses tested (Fig. 6). Compared with the G1 lineage HK/1073 virus, both B Y280 lineage viruses were associated with the highest frequency of virus detection in these tissues and the highest mean titers ($>10^{2.5}$ EID_{50}/ml). However, mice inoculated ocularly with A-L/39 virus did not become

TABLE 4 A(H9N2) virus pathogenicity in mice

Virus and dose[a]	Wt loss[b]	Viral titer ($\log_{10}$ EID$_{50}$/ml) for:[c]			
		Day 3 lung	Day 3 nose	Day 6 lung	Day 6 nose
HK/1073					
10^6	17% (7)	7.6 ± 0.1	3.1 ± 1.0	6.2 ± 0.4	3.4 ± 0.2 (2/3)
10^3		6.4 ± 1.7		6.8 ± 0.5	
Bang/0994					
10^6	2.7% (6)	7.4 ± 0.1	4.2 ± 0.9	6.3 ± 0.1	3.7 ± 0.2
10^3		<1.5 (0/3)		<1.5 (0/3)	
HK/308					
10^6	2.9% (7)	6.6 ± 1.0	4.3 ± 0.5	6.5 ± 1.4 (2/3)	3.2 ± 0.6
10^3		<1.5 (0/3)		<1.5 (0/3)	
A-L/39					
10^6	1.9% (8)	5.3 ± 0.5	4.6 ± 0.1	2.0 (1/3)	4.1 ± 0.9 (2/3)
10^3		2.3 (1/3)		2.3 (1/3)	
ck/VN/70					
10^6	None	2.4 ± 0.5 (2/3)	3.4 ± 1.2 (2/3)	<1.5 (0/3)	<1.5 (0/3)
10^3		<1.5 (0/3)		<1.5 (0/3)	
ck/VN/119					
10^6	None	<1.5 (0/3)	<1.5 (0/3)	<1.5 (0/3)	<1.5 (0/3)
10^3		<1.5 (0/3)		<1.5 (0/3)	

[a]BALB/c mice were intranasally inoculated with 10^6 or 10^3 50% egg infectious doses (EID$_{50}$) of virus in 50 μl as indicated.
[b]Mean maximum percentage weight loss during days 1 to 14 p.i. following inoculation of mice with 10^6 EID$_{50}$ of virus. Day maximum percentage recorded in parentheses. None, viral infection did not lead to weight loss >1%.
[c]Mean viral titer ± standard deviation for all samples with detectable infectious virus ($n = 3$ mice/group unless specified in parentheses). Limit of detection was 1.5 $\log_{10}$ EID$_{50}$/ml.

productively infected (data not shown), demonstrating differences in susceptibility among mammalian models to A(H9N2) virus infection via the ocular route.

Ferret transmissibility of A(H9N2) viruses. A(H9N2) G1 lineage influenza viruses have been previously shown to readily transmit among cohoused ferrets, but not in airborne transmission models (18, 24, 25). In contrast, selected Y280 viruses have

TABLE 5 Pathogenicity and transmissibility of A(H9N2) viruses in ferrets

Experimental group	Wt loss[a]	Temp[b]	Nas dis[c]	Peak NW[d]	Trans[e]	Sero[f]
HK/1073						
Inoculated	6.2 (2/6)	1.7 (6/6)	3/6	6.8 (6/6)	NA	320–640 (3/3)
DC contact	5.0 (1/3)	1.5 (3/3)	0/3	6.5 (3/3)	2/3	320 (2/3)
RD contact	None (0/3)	1.3 (2/3)	0/3	<1.5 (0/3)	0/3	<10 (0/3)
HK/308						
Inoculated	8.4 (4/6)	1.4 (6/6)	1/6	7.8 (6/6)	NA	640–2,560 (6/6)
DC contact	None (0/3)	1.0 (1/3)	0/3	7.8 (3/3)	3/3	640–2,560 (3/3)
RD contact	16.8 (1/3)	None (0/3)	0/3	3.0 (3/3)	2/3	20–40 (2/3)
A-L/39						
Inoculated	11.2 (9/9)	1.6 (9/9)	7/9	8.2 (9/9)	NA	2,560–10,240 (9/9)
DC contact	10.9 (3/3)	2.3 (3/3)	3/3	8.8 (3/3)	3/3	5,120 (3/3)
RD contact	7.8 (3/6)	1.6 (5/6)	0/6	8.0 (5/6)	5/6	2,560–10,240 (5/6)

[a]Percentage mean maximum weight loss during days 1 to 14 p.i./p.c. following inoculation with 10^6 EID$_{50}$/ml of virus. No. of ferrets with weight loss ≥5%/total no. of ferrets is in parentheses.
[b]Mean maximum rise in degrees Celsius above baseline temperature (37.8 to 39.1°C) during days 1 to 14 p.i./p.c. No. of ferrets with temperature rise >1°C/total no. of ferrets in parentheses.
[c]No. of ferrets with detected nasal discharge or sneezing during days 1 to 14 p.i./p.c./total no. of ferrets.
[d]Mean maximum viral titer in ferret nasal wash (NW) specimens expressed as $\log_{10}$ EID$_{50}$/ml. No. of ferrets with detectable virus/total no. of ferrets is in parentheses; limit of detection is $10^{1.5}$ EID$_{50}$/ml.
[e]Transmission (Trans) is shown as the number of contact ferrets with viral titers in NW and seroconversion to homologous virus/total no. of ferrets. NA, not applicable. DC, direct contact; RD, respiratory droplets.
[f]Seroconversion range to homologous virus as determined by HI assay. Titers represent the reciprocal of the dilution that inhibited hemagglutination. No. of ferrets with seroconversion/total no. of ferrets in parentheses. Serum was collected days 17–21 p.i./p.c.
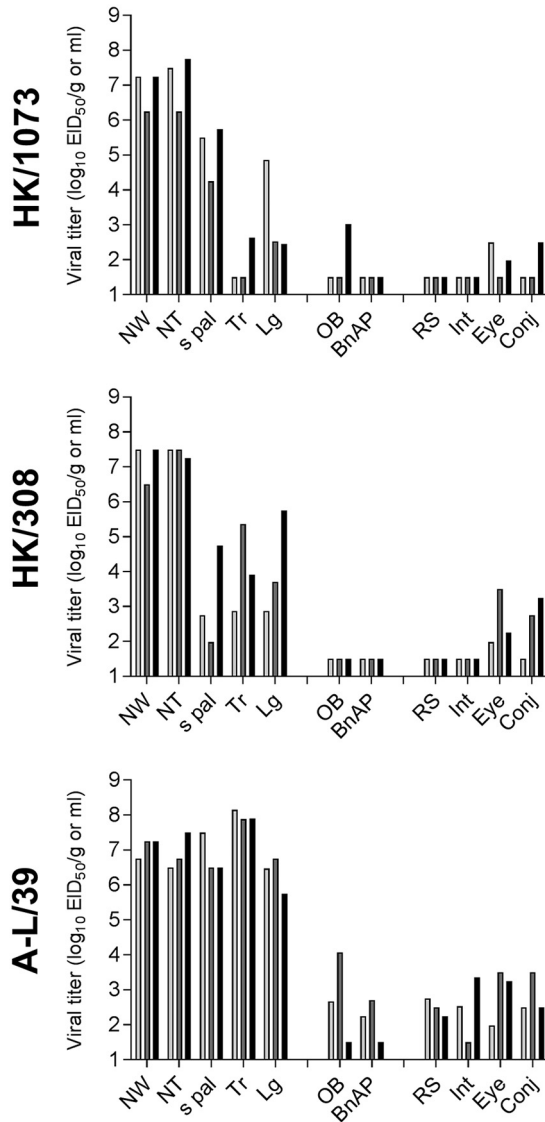
FIG 6 Pathogenicity of A(H9N2) viruses in ferrets. Three ferrets were inoculated intranasally with 10^6 EID_{50} of each virus indicated, and tissues and specimens were collected day 3 p.i. NW, nasal wash; NT, nasal turbinates; s pal, soft palate; Tr, trachea; Lg, lung; OB, olfactory bulb; BnAP, brain (pooled anterior and posterior); RS, rectal swab; Int, intestine (pooled duodenum, jejunoileum, and descending colon); Conj, surrounding conjunctiva. NW, NT, s pal, RS, Eye, and Conj are expressed as EID_{50}/ml of sample or tissue homogenate, and Tr, Lg, OB, BnAP, and Int are expressed as EID_{50}/g. The limit of virus detection was $10^{1.5}$ EID_{50}/g or ml.

demonstrated a capacity for transmission through the air, though these studies have largely been limited to poultry viruses and have not included viruses isolated from humans (4, 26). Here, we assessed the ability of a G1 lineage (HK/1073) and two B Y280 lineage (HK/308, A-L/39) A(H9N2) viruses (inclusive of a range of pH thresholds for HA activation, Table 1) to transmit between ferret pairs using a direct contact (DC) transmission model, which does not restrict the level of exposure between animals, and a respiratory droplet (RD) transmission model, which allows transmission to occur only through the air (Fig. 7). All A(H9N2) viruses that were tested displayed a capacity for transmission between ferret pairs when they were housed together in the same cage (2/3 for HK/1073 virus, 3/3 for HK/308 and A-L/39 viruses). NW samples collected from contact animals were positive for infectious virus as early as 1 day after exposure to inoculated cage mates, and titers reached levels comparable to those for directly inoculated ferrets (Table 5).
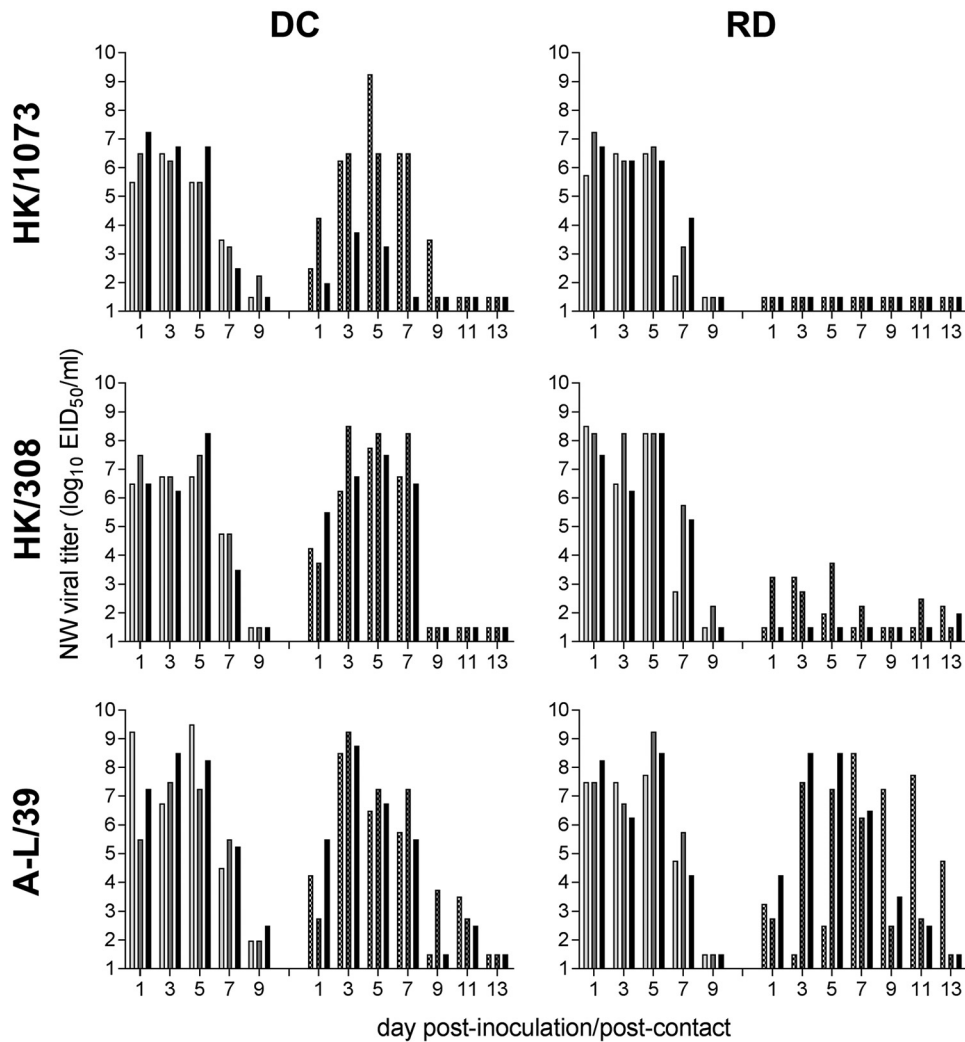
FIG 7 Transmissibility of A(H9N2) viruses between ferrets. Six ferrets were inoculated intranasally with 10^6 EID_{50} of each virus indicated. At 24 h p.i., a naive ferret was placed in the same cage as an inoculated ferret (direct contact [DC] transmission model, left column) or in an adjacent cage with perforated side walls to an inoculated ferret (respiratory droplet [RD] transmission model, right column). Nasal washes (NW) were collected from inoculated (left sets of bars) or contact ferrets (right sets of bars) on alternate days p.i./p.c. Bars represent individual ferrets. The limit of virus detection was $10^{1.5}$ EID_{50}/ml.

During experiments using the more restrictive RD transmission model, HK/1073 virus did not transmit to any of the contact animals (Fig. 7), consistent with previous findings (18). In contrast, A-L/39 transmitted to 3/3 contact ferrets in the initial experiment (Fig. 7) and to 2/3 contact ferrets in a duplicate experiment (summarized in Table 5), resulting in a total of 5/6 contact ferrets shedding infectious virus and with confirmatory seroconversion to homologous virus. HK/308 virus was able to transmit to 2/3 contact ferrets in the RD transmission experiment. Both animals shed detectable virus in NW samples and seroconverted to HK/308 virus, although peak titers were >4 logs lower than those observed in inoculated animals or in the contact animals from the DC transmission experiment (Table 5). Serological titers in convalescent RD contact ferrets (20 to 40) were reduced as well compared to those for the other HK/308 virus-infected animals (640 to 2,560). The third animal in this experiment had trace levels of virus detected in an NW sample 13 days postcontact (p.c.) but did not display seroconversion.

To determine if mammalian adaptive mutations emerged during transmission experiments with A-L/39 virus, NW specimens collected from each transmission pair were

TABLE 6 Amino acid substitutions and frequencies of minor variants observed in ferret nasal wash samples collected during transmission experiments using A/Anhui-Lujiang/39/2018 virus

Experiment	Animal source[b]	PB2	PB1	PA		NP	NS1	HA1[c]			HA2	
							115L/I (84/16)				75T/N (90/10)	
		563Q	617D	532L	466N	253I		39A	78L	224N/K (85/15)		215S
Respiratory droplet transmission experiments												
Ferret pair 1	Inoculated (1 dpi)	Q/R (94/6)	D	L	N	I	L/I (89/11)	A	L	N/K (92/8)	T/N (89/11)	S
Ferret pair 1	Contact (7 dpc)	Q	D	L	N	I	L	A	L	N	T	S
Ferret pair 2	Inoculated (1 dpi)	Q	D	L	N	I	L/I (89/10)	A	L	N/K (81/19)	T/N (88/11)	F
Ferret pair 2	Contact (3 dpc)	Q	D	L	N	I	L	A	L	N	T	S
Ferret pair 3	Inoculated (1 dpi)	Q	D	L	N	I	L/I (88/12)	A	L	N/K (92/8)	T/N (86/14)	S
Ferret pair 3	Contact (3 dpc)	Q	D	L	N	I	L	A	L	K/N (53/46)	T	S
Ferret pair 4	Inoculated (1 dpi)	Q	D	L	N	I/T (94/6)	L/I (88/11)	A	L	N/K (84/16)	T/N (88/12)	S
Ferret pair 4	Contact	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ferret pair 5	Inoculated (1 dpi)	Q	D	L	N	I	L	A	L/R (68/31)	N/K (79/21)	T/N (86/14)	S
Ferret pair 5	Contact (5 dpc)	Q	D	S	N	I	L	A	R	N	T	S
Ferret pair 6	Inoculated (1 dpi)	Q	D	L	N	I	L/I (84/16)	A	L	N/K (91/9)	T/N (84/15)	S
Ferret pair 6	Contact (5 dpc)	Q	D	L	N	I	L	S	L	N	T	S
Direct contact transmission experiments												
Ferret pair 1	Inoculated (1 dpi)	Q	D	L	N	I	L/I (83/17)	A	L	N/K (87/13)	T/N (88/12)	S
Ferret pair 1	Contact (3 dpc)	Q	D	L	N	I	L/I (82/18)	A	L	N/K (93/7)	T	S
Ferret pair 2	Inoculated (3 dpi)	Q	D	L	N	I	L/I (91/8)	A	L	N/K (74/26)	T/N (92/8)	S
Ferret pair 2	Contact (3 dpc)	Q	D	L	N	I	L/I (92/7)	A	L	N/K (94/6)	T/N (59/40)	S
Ferret pair 3	Inoculated (3 dpi)	Q	D	L	N	I	L/I (88/12)	A	L	N/K (78/21)	T/N (88/11)	S
Ferret pair 3	Contact (3 dpc)	Q	D/G (94/6)	L	N/S (94/6)	I	L	A	L/R (76/23)	K/N (54/45)	T/N (95/5)	S

[a]This table presents nonsynonymous changes compared to the inoculum. No amino acid changes or variability was detected for PB1-F2, PA-X, NA, MP, and NEP. Nasal wash samples representing peak titers were chosen for analysis. When mixed populations were detected, the percentage of each amino acid present in the sample is specified in parentheses. ND means that the virus was not detected in this animal during the course of the experiment (limit of detection was $10^{1.5}$ EID$_{50/ml}$).
[b]dpi, days postinoculation; dpc, days postcontact.
[c]Amino acid positions for HA1 are in H3 numbering.

analyzed by deep sequencing. Virus collected from experimentally inoculated ferrets possessed comparable levels of variants as the stock inoculum. Sequence deviations were not consistently identified among infected contact ferrets, though some selection was detected in the contact ferrets in RD transmission experiments (at positions PA-L532S, HA1-A39S, and HA1-L78R) that was not observed in the DC transmission experiments (Table 6). Collectively, these findings indicate that A-L/39 virus possesses an enhanced capacity for mammalian transmissibility compared with other B Y280 or G1 A(H9N2) influenza viruses, while, for the most part, maintaining genetic stability during a ferret-to-ferret transmission event.

DISCUSSION

The continued circulation of A(H9N2) viruses in poultry on multiple continents provides sustained opportunities for the virus to evolve genetically and antigenically, reassort with other avian influenza viruses, and acquire mutations that could enhance mammalian adaptation. Surveillance efforts, such as the active LBM surveillance program in Vietnam described here, play a key role in monitoring contemporary viruses and comparing these poultry isolates to viruses that have jumped the species barrier to cause human infection. While genomic data are essential to initiate viral risk assessment, additional phenotypic studies with isolates of these novel and emerging influenza viruses are crucial. As such, here we have performed a series of *in vitro* and *in vivo* studies to better understand the impact of virus circulation at the animal-human interface and the consequences of A(H9N2) evolution on key factors, including antigenic drift, receptor binding specificity, replication kinetics, pH stability, and mammalian pathogenicity and transmissibility.

The HA genes of A(H9N2) viruses continue to evolve within the Y280 lineage and exhibit considerable genetic diversity among the viruses detected. Importantly, the antigenic drift identified among recent virus isolates from both Y280 lineages warrants additional study to ensure appropriate selection of CVVs and pandemic preparedness (Table 2). Phylogenetic data show that, similar to studies of HPAI A(H5) viruses (27),

A(H9N2) viruses detected in northern border provinces of Vietnam are closely related to those detected in China and indicate that spread of novel viruses from China into Vietnam continues to occur at ports of entry via importation of infected poultry. Still, genetic data from other viruses detected in Vietnam, strikingly similar to viruses detected in China and Myanmar, suggest that some viruses are transported via infected poultry across longer distances. Continued monitoring of borders and ports of entry will help to reduce the introduction and possible emergence of these A(H9N2) viruses into the country. Interestingly, A(H9N2) viruses that have caused human disease in China are genetically very similar to the B Y280 viruses isolated from LBMs in Vietnam, suggesting that viral introductions of these groups of A(H9N2) viruses may also pose a threat to human health in Vietnam. Furthermore, evidence of cross-border spread of a LPAI virus into Vietnam is concerning given the proximity of Vietnam to southern Chinese provinces where LPAI and HPAI A(H7N9) viruses have been detected in previous years. While surveillance for A(H7N9) viruses in Vietnamese LBMs has not detected these viruses, the data in this study suggest that infected poultry and their movements have the potential to introduce other avian influenza subtypes.

Influenza HA protein residues 226 and 228 (mature protein numbering 216 and 218) have been identified as host adaptation sites in A(H2) and A(H3) viruses. While human A(H3) viruses have an L226/S228 combination, avian A(H3) viruses typically have Q226/G228. With the exception of HK/33982 virus, all A(H9) viruses studied here bear the hybrid L216/G218 motif, similar to LPAI A(H7N9) viruses detected in China (L217/G219 by H7 numbering). Three HA residues (180, 216, 217; H3 numbering = 190, 226, 227) have also been the focus of analysis in recent receptor binding studies; for A-L/39 virus, these three positions are T180, L216, and M217. When aspartic acid or glutamic acid were present at position 180, H9 viruses were reported to bind human receptors (8). Alanine and threonine at this position, seen in more recent A(H9) viruses, were also reported to increase binding to human receptors (28). Interestingly, glycan array data for the A-L/39 virus highlighted poor binding to α2,3 and α2,6 glycans. Only the sulfated α2,3 glycan signals were apparent, which is in line with previous reports for A(H9) viruses with these glycans (28). This result was confirmed with recombinant HA. However, conditions in the resialylated RBC assays appeared to overcome the weak binding observed in the glycan microarray experiments. HA interactions with its ligands in glycan-based assays are sensitive to the types of assays employed and experimental conditions. It has been suggested that while real-time binding and enzyme-linked immunosorbent assays (ELISAs) are more sensitive for detecting subtle changes in binding avidity to α2,6 human-type receptors, glycan microarray is a more stringent test of binding specificity (29). The results presented here underscore the diversity of receptor binding capacities of different A(H9N2) viruses associated with human infection and the limitations of any given assay in achieving a complete, comprehensive understanding of this phenotype.

The efficient replication of A(H9N2) viruses in Calu-3 cells observed in this study is in agreement with previous studies employing cells derived from the human respiratory tract. Primary normal human bronchial epithelial (NHBE) cells, related human respiratory cell types, and human bronchus and lung organ cultures support high-titer replication of A(H9N2) viruses, isolated from either humans or avian species, when cultured at 37°C (17, 18, 24, 30), with sustained titers above those of a 2009 A(H1N1) pandemic virus (18, 23). Recent evaluation of A(H5N1) and A(H9N2) reassortant viruses in Calu-3 cells revealed that the presence of A(H9N2) gene segments (notably NS or PB2) conferred a replication advantage compared with the parental A(H5N1) virus, further supporting the high capacity of A(H9N2) viruses to replicate in mammalian cells (31). Similar to other avian influenza virus subtypes, including A(H5N1) and A(H7N9) viruses, all A(H9N2) viruses tested here demonstrated a delay in peak viral titers when Calu-3 cells were cultured at 33°C (19, 32). The robust replication of A-L/39 virus in both human and ferret cells at either 33°C or 37°C was striking, especially as this virus does not possess a lysine at PB2 position 627, which is frequently associated with enhanced replication *in vitro* and *in vivo* (33). In the case of A-L/39 virus, it is possible that the

presence of valine at this position, identified previously as a potential intermediate between 627E and 627K for A(H7N9) viruses, contributes to this phenotype (34, 35). Interestingly, in this experiment a similar effect was not detected for HK/33982 virus, the only virus in this study to bear the compensatory mutation 701N in PB2 (36). Considering differences in replication kinetics observed between selected viruses, further study evaluating polymerase activity in the context of replication capacity would be warranted. As it was difficult to predict *in vivo* replication and transmission of A(H9N2) viruses based on *in vitro* phenotypes and replication efficiency (17), subsequent *in vivo* evaluation of these viruses was necessary.

While LPAI A(H7N9) viruses are capable of causing severe and fatal infection in mice, A(H9N2) virus infection, in general, manifests similarly to other LPAI viruses, in that mild to moderate weight loss (<15%) at high challenge doses is typically observed (18, 24, 37–40). The enhanced capacity of HK/1073 virus to cause disease and replicate efficiently in the murine lung is consistent with these prior studies; notably, the B Y280 lineage viruses that were isolated from humans only caused mild disease in mice (Table 4). A(H9N2) virus replication in nasal washes or nose tissue has been reported previously (4, 37) and supports the frequent detection of selected viruses in this tissue reported here. Reduced infection at lower inoculum doses (10^3 EID_{50}) of recently isolated A(H9N2) viruses is in contrast with many Eurasian avian influenza viruses of the A(H5) and A(H7) subtypes (41, 42). Position 190 in the HA has been implicated in modulating viral replication of A(H9N2) virus in the murine lung, and the presence of threonine or alanine has been shown to diminish virus binding to mouse lung tissue (43), possibly contributing to the reduced replication of some of the Y280 viruses evaluated here. The lack of virus replication in mice following inoculation with ck/VN/119 virus is in line with the poor binding to α2,3-linked sialic acids detected by the hemagglutination assay (Tables 3 and 4). While it appears that several molecular determinants of virulence among A(H9N2) viruses are shared by other virus subtypes (44), additional work is required to better understand the capacity for certain A(H9N2) viruses to cause severe disease in this species (45).

Similar to other avian influenza virus subtypes, A(H9N2) viruses are capable of replication throughout the respiratory tracts of numerous mammalian species following high-dose inoculation (18, 46–49). All A(H9N2) viruses included in this study were detected throughout the ferret respiratory tract on day 3 p.i., consistent with prior studies, albeit showing strain-specific variability in replication and capacity to cause pathological lesions (4, 25, 50). With mean titers of $>10^6$ EID_{50} in nasal turbinates, soft palate, and lung tissues, and mean titers of $>10^7$ EID_{50} in NW and trachea samples at day 3 p.i., A-L/39 virus displayed the most efficient, high-titer replication among all three A(H9N2) viruses examined in ferrets. Furthermore, A-L/39 virus displayed an increased capacity for replication outside the respiratory tract, with higher titers and frequency of detection in brain (including olfactory bulb [OB]), intestinal, and ocular tissues compared with older G1 and B Y280 lineage viruses, as shown here and compared with previous studies (4, 25, 50).

A(H9N2) viruses isolated from mammals are generally capable of transmitting between cohoused ferrets (18, 23, 24), while virus transmission through the air is reportedly rare (4). A-L/39 virus displayed airborne transmission to 5 of 6 animals, mostly within the first day of exposure, despite lacking 627K and 701N in the PB2 protein. Influenza virus transmission is a multifactorial trait. In addition to enhanced polymerase activity, efficient binding to human receptors and a balanced HA and NA, most aerosol-transmissible influenza viruses boast an acid stable HA with a fusion pH threshold below 5.5 (14). It has been speculated that the relatively high fusion threshold (>5.5) for most avian influenza viruses may contribute to the lack of efficient airborne transmission in mammals. Interestingly, we found that the A(H9N2) viruses tested here exhibit a wide pH range for fusion. Many contemporary A(H9N2) viruses, including A-L/39 virus and poultry viruses detected in Vietnamese LBMs, possess a fusion threshold pH typical for human seasonal influenza viruses. As A(H9N2) viruses continue to accumulate molecular changes that further adapt them to the human host,

the likelihood of the emergence of this virus subtype in susceptible populations increases. Our findings show the importance of surveillance activities in both poultry populations and humans to detect the emergence of zoonotic influenza viruses that pose a threat to public health. Continued study of A(H9N2) human viruses and the identification of genetic and phenotypic similarities they share with circulating avian viruses is critical for early detection of influenza viruses capable of zoonotic infections and potential pandemics.

MATERIALS AND METHODS

Surveillance. LBM surveillance performed by the Department of Animal Health, Vietnam (Ministry of Agriculture and Rural Development), was conducted from September 2017 to September 2018 in 12 provinces. Provinces met specific criteria for LBM sampling, including reporting previous poultry outbreaks and having avian influenza prevalence in LBMs of >25% during the prior 2-year period, sharing a border with another country, having a high poultry population density, and high demand for poultry consumption. Five markets were selected per province. Samples were collected monthly per market from 6 poultry vendors selected at random; 5 birds were randomly swabbed (oropharyngeal) from each vendor and pooled (6 pooled samples/market). Environmental samples were also collected from each market with 5 fecal samples pooled into 1 sample and 5 water samples pooled into 1 sample. In total, 5,760 samples were collected in the 1-year period. Influenza A-positive samples, as determined by universal M gene real-time reverse transcription-PCR (rRT-PCR), were subjected to genomic sequencing using previously described methods (51).

Viruses. Influenza A(H9N2) viruses identified in Vietnam LBMs are listed in Table S1, and those phenotypically characterized in this study are listed in Table 1. LPAI A(H7N9) A/Shanghai/1/2013 (Shanghai/1), seasonal A(H3N2) A/Switzerland/9715293/2013 (Switz/13), and 2009 pandemic A(H1N1) A/California/7/2009 (Cal/7) were also included in selected assays. All A(H9N2) and A(H7N9) viruses were propagated in the allantoic cavity of 10- to 11-day-old embryonated chicken eggs (Hyline) at 37°C for 48 h as described previously (42). A(H1) and A(H3) viruses were propagated in Madin-Darby canine kidney (MDCK) cells as described previously (52). Pooled allantoic fluid or cell culture supernatant was clarified by centrifugation and frozen in aliquots at −80°C until use, and stock 50% egg infectious dose (EID_{50}) or PFU titers were determined. All experiments were conducted under biosafety level 3 conditions including enhancements required by the U.S. Department of Agriculture (53). GISAID accession numbers of the viruses sequenced for this study are included in Table S1, with viruses chosen for *in vitro*/*in vivo* analysis in boldface type.

Phylogenetic tree construction and molecular analysis. A total of 832 A(H9N2) HA sequences from GISAID/GenBank databases were used to construct HA trees. Data were aligned via MUSCLE (54), and sequences were trimmed to the beginning of the mature HA1 protein gene sequence using BioEdit v7.0. The neighbor-joining method (55) with the Jukes-Cantor model in the MEGA 7.0 software package (56) were used to construct the final tree. The percentage of replicate trees in which the associated taxa clustered together was measured using the bootstrap test (1,000 replicates), and bootstrap values are shown next to the branches. Nucleotide sequence alignments used for the HA trees, as well as NA and internal gene alignments, were translated to amino acid protein sequences to compare the amino acid sequences among the virus.

Reassortment analysis. Full genome sequences of A(H9N2) influenza viruses were used to identify potential reassortment events by aligning individual gene segments from this study to the top 100 BLAST hits in the GISAID database using Clustal W (57). Phylogenetic trees of all 8 segments were built using the MrBayes software (58) and screened for reassortment using the GiRaF version 1.09 software (59).

Antigenic characterization. A(H9N2)-positive samples were inoculated into 10- to 11-day-old embryonated chicken eggs or MDCK cells, and allantoic fluid or supernatants were harvested 48 h postinoculation. Isolation-positive samples ($n = 34$) were identified by hemagglutination assay using turkey red blood cells. Antigenic characterization of virus isolates was performed using the hemagglutination inhibition assay with polyclonal ferret immune sera raised against representative viruses and CVVs, as previously described (60).

Virus-induced syncytium formation assay for HA fusion pH measurement. Infection of Vero cells (ATCC) with influenza A viruses was used to determine the highest pH value at which syncytia were observed. As described previously (15), infected cell cultures were incubated with fusion buffer adjusted to a 5.2 to 6.0 range of pH values in 0.1-unit increments and were fixed and stained using anti-NP antibody 3 h after fusion induction. The fusion pH threshold is defined as the highest pH value at which 50% or more syncytia can be achieved.

HA receptor binding specificity. Hemagglutination assays using resialylated turkey red blood cells were performed as previously described (61). Control viruses for this assay included beta-propiolactone (BPL)-inactivated A/mallard/Netherlands/12/2000 A(H7N7) (ATCC) ($\alpha 2,3$ binding control), A/Washington/74/2009 A(H1N1) ($\alpha 2,6$ binding control), and A/pheasant/New Jersey/1355/1998-PR8 A(H5N2) (ATCC) (dual $\alpha 2,3$ and $\alpha 2,6$ binding control).

Recombinant HA cloning, expression, and purification. The cDNA encoding the mature ectodomain of A-L/39 virus was synthesized as codon-optimized construct (Genscript, Inc.) and subcloned into the baculovirus transfer vector pAcGP67-B (BD Biosciences) in frame with an N-terminal baculovirus GP67 signal peptide and a T4 fibritin sequence for generating functional trimers, and a His tag to aid purification (62). Transfection and virus amplification were carried out using AB Vector's (San Diego, CA)

linearized baculovirus vector DNA and their transfection protocols. Secreted soluble recombinant HA protein was recovered from the cell culture supernatant by tangential flow filtration through a 30-kDa-molecular-weight-cutoff membrane, metal affinity chromatography, and gel filtration chromatography. Recombinant HA of A/Vietnam/1203/2004 (Viet04) and A/Switzerland/9715293/2013 has been described elsewhere (63, 64).

Glycan binding studies. Glycan microarray slides were produced under contract from the Centers for Disease Control and Prevention by James Paulson at The Scripps Research Institute (La Jolla, CA) (see Table S2 for glycans present on the array). BPL-inactivated viruses were diluted in phosphate-buffered saline (PBS) with 2% wt/vol bovine serum albumin (PBS-BSA) to an HA titer of 128. Viruses were applied to the slides and incubated at 4°C with gentle agitation for 1.5 h. Slides were rinsed with PBS with 0.05% Tween 20 (PBS-T) and PBS to remove unbound virus. The slides were then incubated sequentially with ferret serum (90 min; 1/1,000 dilution with PBS) raised against A(H9) viruses (sera used with each virus specified in Fig. 2), a biotinylated goat anti-ferret IgG antibody (30 min; 1/200 dilution with PBS) (Rockland), and with streptavidin-Alexa Fluor488 conjugate (30 min; $1/2$,000 dilution with PBS) (Thermo Fisher, USA), with brief PBS-T/PBS washes being performed after each incubation. After the final PBS-T/PBS wash, the slides were briefly washed in deionized water, dried by a gentle steam of nitrogen gas, and immediately subjected to imaging. Fluorescence intensities were detected using an Innoscan 1100AL scanner (Innopsys, USA). Image analyses were carried out using ImaGene 9 image analysis software (BioDiscovery, Inc., USA).

Recombinant HA glycan microarray analyses were performed as described previously (21, 52). Briefly, recombinant HA-antibody complexes were prepared by mixing rHA, mouse anti-penta-His-Alexa Fluor 488 (Qiagen), and anti-mouse-IgG-Alexa Fluor 488 (Thermo Fisher Scientific) in a molar ratio of 4:2:1, respectively. Prepared complexes were incubated for 1 h at 4°C, diluted to 0.5 ml with PBS-BSA, and incubated on the microarray slide in a 4°C humidified chamber for 1.5 h. Slides were subsequently washed by successive rinses in PBS-T, PBS, and deionized water and then immediately subjected to imaging. Fluorescence intensities were detected using an Innoscan 1100AL scanner (Innopsys, USA). Image analyses were carried out using ImaGene 9 image analysis software (BioDiscovery, Inc., USA). The glycans used in these experiments are listed in Table S2.

***In vitro* experiments.** The human bronchial epithelial cell line Calu-3 (ATCC) was propagated and cultured as described previously (19). Cells were seeded on 12-well semipermeable inserts (Corning) and cultured with apical and basolateral media. Following washing, Calu-3 cells were inoculated apically at a multiplicity of infection (MOI) of 0.01 (based on stock PFU titer) in serum-free medium, and incubated for 1 h before washing and subsequent culture at either 37°C or 33°C. Culture supernatant aliquots were collected at the indicated times p.i. and frozen at −80°C until titration in MDCK cells for determination of infectious titer (PFU/ml).

FNEC and FTEC were isolated and cultured as previously described (65, 66). Cells were grown on 12-well inserts at air-liquid interface (ALI) conditions and were inoculated apically at an MOI of 0.01 in serum-free medium and incubated for 1 h before washing and subsequent culture at either 37°C or 33°C. Cells remained under ALI conditions for the duration of the experiment, with basolateral medium changed every-other-day. For sample collection, 200 μl medium was added apically for a 10-min incubation prior to collection and freezing at −80°C until titration in MDCK cells for determination of infectious titer (PFU/ml). The statistical significance of all viral replication kinetics experiments was assessed using two-way analysis of variance with a Tukey posttest.

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 weeks of age, were anesthetized with vaporized isoflurane and inoculated by the intranasal route with 10^6 or 10^3 EID$_{50}$ of the indicated A(H9N2) viruses in a 50-μl volume. Five mice per group inoculated with 10^6 EID$_{50}$ of virus were observed daily for morbidity and mortality. Three mice per group inoculated with either dose were euthanized at day 3 or 6 p.i. for collection of nose and lung tissue for determination of virus replication by titration of homogenized tissues in eggs (limit of detection, $10^{1.5}$ EID$_{50}$/ml). Eight additional mice were anesthetized as described above and inoculated by the ocular route with 10^6 EID$_{50}$ of A-L/39 virus in a 5-μl volume as described previously (67); four mice were euthanized at day 3 or 6 p.i. for collection of eye, nose, and lung tissue for determination of virus replication (limit of detection, $10^{0.8}$ EID$_{50}$/ml).

Ferret experiments. Male Fitch ferrets (Triple F Farms), 6 to 11 months of age and serologically negative by a standard hemagglutination assay for currently circulating A(H1N1), A(H3N2), and B influenza viruses, were employed in this study. Ferrets were housed in a Duo-Flo Bioclean mobile environmental enclosure (Lab Products) for the duration of each experiment. Ferrets were anesthetized intramuscularly with a ketamine cocktail and inoculated intranasally with 10^6 EID$_{50}$ of each A(H9N2) virus diluted in PBS in a 1-ml volume. Three ferrets per group were observed daily for clinical signs of infection, with nasal washes, conjunctival wash, and rectal swabs collected on alternate days p.i. for determination of virus shedding as previously described (68). Three additional ferrets per group were euthanized on day 3 p.i. for assessment of virus replication and systemic spread of virus, with sample titration of tissue specimens performed as described above.

Virus transmissibility was assessed by placing a serologically naive ferret in the same cage as an inoculated ferret (to assess virus transmission in the presence of direct contact [DC]) or in an adjacent cage with perforated side walls to allow air exchange in the absence of direct or indirect contact between animals (to assess virus transmission by respiratory droplets [RD]) as previously described (69). Note that the DC transmission model provides the opportunity for transmission to occur via contact or

inhalation of airborne viruses, while the RD transmission model restricts transmission events to those occurring through the air. Serum was collected on days 18 to 21 p.i./postcontact (p.c.) to measure seroconversion against homologous virus by standard hemagglutinin inhibition assay with 0.5% turkey red blood cells.

Next-generation sequencing. Viral RNA from ferret nasal wash specimens (the collection day associated with highest infectious viral load per ferret) was extracted with a QIAamp viral RNA minikit (Qiagen) per the manufacturer's instructions. The codon complete influenza virus genomes were amplified and deep sequenced on an Illumina MiSeq instrument as described previously (70).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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