Evaluation of the Biological Properties and Cross-Reactive Antibody Response to H10 Influenza Viruses in Ferrets

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ABSTRACT The recent outbreak of avian origin H10N7 influenza among seals in northern Europe and two fatal human infections with an avian H10N8 virus in China have demonstrated that H10 viruses can spread between mammals and cause severe disease in humans. To gain insight into the potential for H10 viruses to cross the species barrier and to identify a candidate vaccine strain, we evaluated the *in vitro* and *in vivo* properties and antibody response in ferrets to 20 diverse H10 viruses. H10 virus infection of ferrets caused variable weight loss, and all 20 viruses replicated throughout the respiratory tract; however, replication in the lungs was highly variable. In glycan-binding assays, the H10 viruses preferentially bound “avian-like” α 2,3-linked sialic acids. Importantly, several isolates also displayed strong binding to long-chain “human-like” α 2,6-linked sialic acids and exhibited comparable or elevated neuraminidase activity relative to human H1N1, H2N2, and H3N2 viruses. In hemagglutination inhibition assays, 12 antisera cross-reacted with ≥ 14 of 20 H10 viruses, and 7 viruses induced neutralizing activity against ≥ 15 of the 20 viruses. By combining data on weight loss, viral replication, and the cross-reactive antibody response, we identified A/mallard/Portugal/79906/2009 (H10N7) as a suitable virus for vaccine development. Collectively, our findings suggest that H10 viruses may continue to sporadically infect humans and other mammals, underscoring the importance of developing an H10 vaccine for pandemic preparedness.

IMPORTANCE Avian origin H10 influenza viruses sporadically infect humans and other mammals; however, little is known about viruses of this subtype. Thus, we characterized the biological properties of 20 H10 viruses *in vitro* and in ferrets. Infection caused mild to moderate weight loss (5 to 15%), with robust viral replication in the nasal tissues and variable replication in the lung. H10 viruses preferentially bind “avian-like” sialic acids, although several isolates also displayed binding to “human-like” sialic acid receptors. This is consistent with the ability of H10 viruses to cross the species barrier and warrants selection of an H10 vaccine strain. By evaluating the cross-reactive antibody response to the H10 viruses and combining this analysis with viral replication and weight loss findings, we identified A/mallard/Portugal/79906/2009 (H10N7) as a suitable H10 vaccine strain.

KEYWORDS avian viruses, influenza, influenza vaccines

Influenza pandemics occur when an antigenically novel influenza virus is introduced and spreads in the human population. Influenza A viruses belong to the family *Orthomyxoviridae* and are divided into subtypes based on their major surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Aquatic waterfowl are the natural hosts and reservoir for influenza A viruses, and 16 HA and 9 NA subtypes circulate in these birds (1–3). Of the HA subtypes, H1, H2, H3, H7, and H10 viruses have caused large outbreaks with onward transmission in mammals. Influenza pandemics

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have been caused by viruses of the H1N1, H2N2, and H3N2 subtypes, and H1 and H3 subtype viruses circulate in pigs worldwide (4). In horses, H7N7 and H3N8 viruses were panzootic and cocirculated until the late 1980s, when H3N8 became the dominant subtype (5).

The first H10 influenza virus was isolated from a chicken in Germany in 1949 [A/chicken/Germany/N/1949 (H10N7) (ck/GM/49)] (6), and phylogenetically, H10 viruses fall into distinct Eurasian and North American lineages (7, 8). The first report of interspecies transmission of an H10 virus occurred in 1984, when an avian H10N4 virus was introduced into farmed mink in Sweden. This outbreak involved ~100,000 animals, and infection resulted in almost 100% morbidity with 3,000 deaths (9–11). Follow-up studies determined that the virulence in mink was associated with mutations in the viral nonstructural gene 1 (NS1) that significantly impaired the host antiviral response (8, 12).

In 1986 and 1993, H10N4 and H10N1 viruses were isolated from farmed turkeys and ducks in Great Britain and Singapore, respectively. These viruses caused lethal infections in poultry and were defined as highly pathogenic (HP) using the intravenous pathogenicity index (IVPI), despite the absence of a polybasic cleavage site in the HA (13, 14). In 2007 and in 2009 and 2010, additional H10 viruses were isolated in Italy and China that also fulfilled these criteria (15, 16). Pathogenesis studies with the isolate from 2007 determined that lethality and virulence in poultry were the result of high levels of viral replication in the kidneys (15), a pattern that was quite distinct from that of the multisystem hemorrhagic disease seen with H5 and H7 HP avian influenza viruses.

The first reported human infections with an H10 virus occurred in Egypt in 2004 (17). Two infants presented with a fever and cough, and H10N7 viruses were isolated from both patients. In subsequent surveillance studies, 5 closely related viruses were isolated from hunter-killed wild ducks sold at bird markets (17). Four years later, an H10N5 virus was isolated in China from a pig at slaughter (18), and in 2010 in Australia, H10N7 viruses were transmitted from poultry to 7 abattoir workers causing conjunctivitis and minor upper respiratory tract symptoms (19). While there was no evidence of seroconversion of the infected abattoir workers, a seroprevalence study of turkey farmers in the United States identified a small number of farmers with antibodies against H10 influenza viruses (20). These studies suggest that sporadically, humans are occupationally exposed to H10 viruses.

Consistent with these reports, an H10N8 virus emerged in China in 2013 and infected three people who had visited live-bird markets (21–23). All three patients developed severe disease, and two died (22). The following year, in response to mass seal die-offs in Sweden, Denmark, and Germany (24–26), animals were necropsied and an H10N7 virus was identified as the causative agent. Importantly, these recent reports establish that avian H10 viruses can cause severe disease in humans and transmit among mammalian species. Thus, to gain further insight into the potential for H10 viruses to cross the species barrier and to identify a suitable H10 vaccine strain, we characterized 20 isolates that geographically and temporally span the breadth of H10 subtype viruses and performed cross-hemagglutination inhibition (HI) and neutralization assays using postinfection ferret antiserum to identify a candidate vaccine strain.

RESULTS

Virus strain selection. To identify a representative panel of viruses that encompass the H10 subtype, we performed phylogenetic analysis of the HA gene. Consistent with those of other subtypes, H10 virus HAs form two distinct lineages: North American and Eurasian (Fig. 1). From this analysis, viruses were selected to represent diverse geographical origins, years of isolation, and avian hosts and included all 9 NA subtypes. When possible, viruses that had transmitted to mammals [e.g., A/mink/Sweden/E12265/1984 (H10N4) (mk/SW/84)] or were direct precursors of the viruses that transmitted to mammals [e.g., A/chicken/New South Wales/D809/2010 (H10N7) (ck/NSW/10)] were included. The list of viruses is displayed in Table 1, and Fig. 1 shows an abbreviated phylogenetic tree that includes these viruses. In addition, although the HA

FIG 1 Phylogenetic relationships among the selected H10 influenza viruses. The evolutionary history of the H10 hemagglutinins was inferred by the maximum-likelihood method, and evolutionary distances were calculated using the Tamura-Nei model with 500 bootstrap replicates. The tree was generated using the maximum-composite-likelihood (MCL) approach with MEGA7. The tree is drawn to scale, and the phylogenetic distance of each sequence from the nearest node is shown in parentheses. #, isolates included in the phylogenetic tree that were not included in further analysis. A/duck/Manitoba/1953 (H10N7) is the first H10 isolate from North America, and A/swine/Hubei/10/2008 (H10N5) is the only H10 isolate from pigs. These isolates are included in the tree to illustrate relationships between the viruses and the panel of H10 viruses. The sequences for A/mandarin duck/Singapore/805/F-72/7/1993 (H10N5) and A/turkey/Minnesota/5/1979 (H10N7) were not available during the initial phylogenetic analysis for strain selection; however, after obtaining these isolates, the HA gene was sequenced, and they were included in the phylogenetic analysis to show the relationships among all the viruses studied.

sequence for A/mandarin duck/Singapore/805/F-72/7/1993 (H10N5) (mdk/SG/93) had not been published, the virus was included due to its highly pathogenic phenotype as determined by the IVPI. We also included an early North American lineage virus, A/turkey/Minnesota/5/1979 (H10N7) (tk/MN/79) from the NIAID influenza virus collection. In total, 20 H10 viruses were included, from the earliest isolate (1949) to the most recent human (2013) and seal isolates (2014).

H10 virus replication and weight loss in ferrets. We first sought to evaluate the H10 viruses in ferrets, which are a well-recognized model of influenza virus infection because they develop clinical signs (lethargy, nasal discharge, and weight loss) and shed high titers of virus following infection with human influenza viruses (27). Weight loss was strain specific and highly variable (Fig. 2 and 3), with no obvious differences between the North American and Eurasian lineage viruses. However, three viruses, ck/NSW/10, mk/SW/84, and A/mallard/Portugal/79906/2009 (mal/PT/09), caused $\geq 10\%$ weight loss lasting more than 2 days in at least 2 animals. Of interest, neither mdk/SG/93, which is lethal in chickens, nor the recent H10 isolates JX/13 and A/seal/Germany/EMC-1/2014 (sl/GM/14), which transmitted to and caused severe disease in humans and seals, respectively, caused substantial weight loss or severe disease in ferrets.

On days 3 and 5 postinfection, we evaluated viral replication in the nasal turbinates, lungs, and brains of the ferrets (Fig. 4). In the nasal turbinates (Fig. 4A), all the H10 viruses grew to titers of $>10^4$ 50% tissue culture infective doses (TCID₅₀)/g. The majority of viruses achieved titers of $>10^6$ TCID₅₀/g on both days, except A/American black duck/New Brunswick/00477/2010 (bldk/NB/10) and A/quail/Italy/1117/1965 (ql/IT/65), which achieved titers of $>10^6$ TCID₅₀/g only on days 5 and 3, respectively. Interestingly, the earliest H10 isolate, ck/GM/49, did not replicate to titers of $>10^4$

TABLE 1 H10 influenza viruses evaluated and abbreviated names

Virus	Abbreviation	Subtype	Passage history ^c	Mammalian mutation(s) in PB2 gene segment
North American lineage				
A/turkey/Minnesota/5/1979	tk/MN/79	H10N7	E1/E2	Sequence not available
A/ruddy turnstone/New Jersey/471613/2001	rt/NJ/01	H10N7	E2/E2	
A/northern shoveler/Washington/44249-675/2006	nshov/WA/06	H10N2	E1/E2	
A/mallard/Minnesota/SG057/2007	mal/MN/07	H10N7	E1/E2	
A/ruddy turnstone/New Jersey/SG-498/2008	rt/NJ/08	H10N7	E1/E2	
A/shorebird/Delaware Bay/139/2009	sb/DE/09	H10N7	E1/E2	
A/northern pintail/Interior Alaska/9BM5376R0/2009	npin/AK/09	H10N7	E1/E2	
A/American black duck/New Brunswick/00477/2010	bldk/NB/10	H10N6	EX/E2	
A/chicken/New South Wales/D809/2010 ^a	ck/NSW/10	H10N7	C1/E2	
Eurasian lineage				
A/chicken/Germany/N/1949	ck/GM/49	H10N7	EX/E2	E627K ^d
A/quail/Italy/1117/1965	ql/IT/65	H10N8	EX/E2	
A/duck/Hong Kong/562/1979	dk/HK/79	H10N9	EX/E2	K526R, D701N ^d
A/mink/Sweden/E12665/1984	mk/SW/84	H10N4	EX/E2	
A/mandarin duck/Singapore/805/F-72/7/1993 ^b	mdk/SG/93	H10N5	E1/E2	Sequence not available
A/mallard/Sweden/770/2002	mal/SW/02	H10N6	EX/E2	
A/Eurasian wigeon/Netherlands/4/2007	wig/NL/07	H10N1	E1/E2	Sequence not available
A/mallard/Portugal/79906/2009	mal/PT/09	H10N7	E1/E2	
A/duck/Thailand/LM-CU4775/2009	dk/TH/09	H10N3	C2/E2	E627K (H9N2 internal protein genes) ^e
A/Jiangxi-Donghui/346/2013	JX/13	H10N8	C1/E2	
A/seal/Germany/EMC-1/2014	sl/GM/14	H10N7	C1	

^aVirus was isolated from poultry during an outbreak that resulted in human infections, and the virus is closely related to A/Sydney/1/2010 (H10N7). A/Sydney/1/2010 was not successfully isolated during the outbreak; however, partial sequences for HA were recovered (19).

^bVirus is highly pathogenic as defined by the IVP; however, the HA does not have a polybasic cleavage site (14).

^cE, egg passage; C, MDCK cell passage; X, unknown number of passages. Passage history is indicated as passage received/subsequent passage used in experiments. For example, E1/E2 indicates that passage E1 was received, and the virus was passaged two additional times in eggs (E2) for use in experiments.

^dMutation was described previously (8).

^eMutation and internal gene origin were described previously (21).

TCID₅₀/g on either day. In the lungs (Fig. 4B), we observed much greater variation between animals and between days 3 and 5 for a given virus. Also, viral replication was highly variable between different H10 isolates, with no obvious differences between the North American and Eurasian lineage viruses. Notably, bldk/NB/10, ql/IT/65, and ck/GM/49, which grew poorly in nasal turbinates, also did not replicate to high titers in the lung. The human isolate JX/13, which grew to high titers in nasal turbinates, replicated poorly in the lungs. Isolates ck/NSW/10 and mk/SW/84 grew to the highest titers in the lungs among the H10 viruses on both study days, although the titers for mk/SW/84 were variable between animals on day 5.

Most of the H10 viruses did not replicate in the brain (Fig. 4C). However, low-level replication of tk/MN/79, A/northern shoveler/Washington/44249-675/2006 (nshov/WA/06), A/shorebird/Delaware Bay/139/2009 (sb/DE/09), ck/NSW/10, and A/duck/Hong Kong/562/1979 (dk/HK/79) was detected in a proportion of the inoculated ferrets. It was not possible to accurately determine whether A/ruddy turnstone/New Jersey/471613/2001 (rt/NJ/01), A/mallard/Minnesota/SG057/2007 (mal/MN/07), A/ruddy turnstone/New Jersey/SG-498/2008 (rt/NJ/08), and A/mallard/Sweden/770/2002 (mal/SW/02) replicated in the brain because the olfactory bulb was not separated from the brain during tissue collection and homogenization. Collectively, these findings indicate that H10 viruses grow to high titers in nasal turbinates, with highly variable replication in the lungs and minimal replication in the brain.

Glycan-binding specificity of H10 viruses and sequence analyses. As receptor-binding specificity is a determinant of the host range, we next evaluated the binding preferences of the H10 viruses in a solid-phase binding assay with α 2,3- and α 2,6-linked sialic acids of various lengths. We included well-characterized receptor-binding variants (HA 226Q and 228G [QG] and 226L and 228S [LS]) of A/Japan/305/1957 (H2N2) influenza viruses on the cold-adapted (ca) A/Ann Arbor/6/1960 vaccine backbone as

FIG 2 Weight loss in ferrets infected with North American lineage H10 influenza viruses. Ferrets ($n = 2$ or 3 /virus) were inoculated intranasally with 10^6 TCID₅₀ of each H10 virus in a 1-ml inoculum. The animals were weighed daily to determine percent weight loss.

controls (28). As expected, the QG and LS H2N2 ca viruses exhibited strong binding preferences for α 2,3- and α 2,6-linked sialic acids, respectively (Fig. 5). All 20 H10 viruses exhibited a binding preference for α 2,3-linked sialic acids across the three chain lengths (Fig. 5), and regardless of receptor-binding preference, the virus binding signal increased with sialic acid chain length for both the control and H10 viruses. Two isolates, ck/GM/49 and mal/PT/09, displayed exclusive α 2,3-linked sialic acid binding; however, several viruses also showed marked binding to α 2,6-linked sialic acids, and six viruses, rt/NJ/01, rt/NJ/08, ck/NSW/10, ql/IT/65, mk/SW/84, and sl/GM/14, had binding signals for long-chain 6'SLN-LN (see Materials and Methods) that exceeded 50% of the maximal binding signal. In particular, ck/NSW/10 and ql/IT/65 exhibited the highest degree of binding to 6'SLN-LN and 6'SLN-LN-LN, and ql/IT/65 had binding signal intensities for 3'SLN-LN and 6'SLN-LN and for 3'SLN-LN-LN and 6'SLN-LN-LN that were equivalent at each chain length.

To identify specific amino acids or mutations that may be associated with binding to α 2,6-linked sialic acids, we analyzed the HA sequences for the panel of H10 viruses. The regions of HA that form the receptor-binding pocket or site consist of the 130 loop, 150 loop, 190 helix, and 220 loop (29). In our sequence analyses, all the H10 viruses had 226Q and 228G, consistent with a binding preference for α 2,3-linked sialic acids. There were no shared amino acid sequences among the viruses that displayed pronounced α 2,6-linked sialic acid binding; however, both ql/IT/65 and ck/NSW/10, which displayed the highest degree of α 2,6-linked sialic acid binding, had several substitutions in key regions of the receptor-binding site (Table 2). For ql/IT/65, these mutations included

FIG 3 Weight loss in ferrets infected with Eurasian lineage H10 influenza viruses. Ferrets ($n = 2$ or 3 /virus) were inoculated intranasally with 10^6 TCID₅₀ of each H10 virus in a 1-ml inoculum. The animals were weighed daily, and percent weight loss is presented. Due to the reported lethality of mk/SW/84 in mink, an initial experiment was performed in which one ferret was infected with 10^4 TCID₅₀ and a second ferret was infected with 10^6 TCID₅₀. After determining that the virus was not lethal in ferrets, two additional ferrets (broken lines) were infected at a dose of 10^6 TCID₅₀ and weighed every other day.

129P and 138S, 157G, and 217I (H3 numbering) in the 130, 150, and 220 loops, respectively, and for ck/NSW/10, there were single amino acid mutations at 137S and 193D in the 130 loop and 190 helix (Table 2).

NA activity of H10 viruses. As an increase in NA activity has been shown to promote airborne transmission of pandemic H1N1 viruses (30) and balanced HA and NA activities are considered a requirement for the emergence of a pandemic influenza virus (31), we evaluated the NA activity of the H10 viruses in parallel with three human influenza isolates. The human viruses were A/California/07/2009 (H1N1pdm09), A/Perth/16/2009 (H3N2), and A/Japan/305/1957 (H2N2). Importantly, these viruses exhibited a relatively narrow range of NA activity, with A/Perth/16/2009 (H3N2) and A/Japan/305/1957 (H2N2) displaying the highest and lowest activities, respectively (Fig. 6), and A/California/07/2009 (H1N1pdm09) exhibiting intermediate activity. Three H10 viruses (rt/NJ/08, ck/NSW/10, and mal/SW/02) had NA activity that was comparable to that of the human viruses, and mk/SW/84 had elevated activity relative to all the viruses examined. dk/HK/79 and mal/MN/07 also exhibited NA activity that was elevated compared to that of the human viruses. At the highest virus concentrations, tk/MN/79, sb/DE/09, and A/Eurasian wigeon/Netherlands/4/2007 (wig/NL/07) also exhibited higher activity than the human viruses. The remaining 11 H10 viruses had relatively low NA activity, and sl/GM/14 had the lowest NA activity of all the viruses examined.

Cross-reactive HI and neutralizing-antibody response. To determine the cross-reactivity of the antibody response, postinfection sera were raised against all 20 viruses in ferrets, and HI assays were performed against the panel of H10 viruses. Several of the antisera had homologous titers of $<1:80$ (not shown), so sera from all the animals that

FIG 4 Replication of H10 influenza viruses in the nasal turbinates, lungs, and brains of ferrets. Ferrets ($n = 2$ or 3 /virus) were inoculated intranasally with 10^6 TCID₅₀ of each H10 virus. On days 3 and 5 postinfection, tissues were collected, homogenized, and titrated on MDCK cells. Shown are viral titers in nasal turbinates (A), lungs (B), and brains (C). The symbols represent the titers from individual animals, with the means indicated by horizontal lines. For rt/NJ/01, rt/NJ/08, mal/MN/07, and mal/SW/02, brain tissue was collected without removing the olfactory bulb; thus, the titers for those isolates may not accurately reflect replication in the brain.

were infected with a given virus were pooled and concentrated (~ 10 -fold). The results of the cross-HI assays are presented in Table 3. Antibody titers ≤ 4 -fold below the homologous antibody titer were considered cross-reactive, while titers below this threshold indicated a lack of reactivity. Of the 20 sera evaluated, 12 cross-reacted with ≥ 14 of the H10 viruses. Antisera raised against tk/MN/79, A/northern pintail/Interior Alaska/9BM5376R0/2009 (npin/AK/09), ck/NSW/10, ck/GM/49, and sl/GM/14 cross-reacted with fewer than 3 other viruses. Of interest, the antiserum against sl/GM/14 failed to cross-react with any of the H10 viruses.

Subsequently, 10 antisera that exhibited cross-reactivity by HI assay (i.e., $\geq 14/20$ viruses) were subjected to microneutralization assays against all 20 H10 viruses (Table 4). Antisera against bldk/NB/10 and qI/IT/65 were excluded from further analysis because they replicated poorly in ferrets (Fig. 4). We found that concentrated sera used in the HI assays had high neutralizing titers (not shown); thus, the neutralizing-antibody assays were performed with unconcentrated sera. Using the same cutoff of ≤ 4 -fold below the homologous antibody titer indicating cross-reactivity, the HI and neutralization assays exhibited a high degree of concordance; 7 of the 10 antisera that exhibited cross-reactive HI titers cross-neutralized ≥ 15 of the H10 viruses. Postinfection sera against sb/DE/09, wig/NL/07, and A/duck/

FIG 5 Receptor-binding preferences of H10 influenza viruses determined by solid-phase binding assay. To determine the receptor-binding preferences, H10 influenza viruses were incubated on 384-well plates coated with α 2,3- and α 2,6-linked sialic acids. The panel of glycans included 3'SLN, 3'SLN-LN, 3'SLN-LN-LN, 6'SLN, 6'SLN-LN, and 6'SLN-LN-LN. H10 and H2 viruses were detected using goat polyclonal H2- and H10-specific hyperimmune sera with a secondary anti-goat HRP-conjugated antibody. Control viruses included A/Japan/305/1957 (H2N2) cold-adapted vaccine viruses carrying the QG or LS receptor-binding mutations. Shown are the glycan-binding profiles of H10 viruses from the North American (A) and Eurasian (B) lineages. For clarity, the viruses were divided by lineage, and the findings for the control viruses are presented in both panels. The error bars indicate standard errors of the means.

Thailand/LM-CU4775/2009 (dk/TH/09) that cross-reacted with ≥ 14 viruses by HI neutralized fewer than half of the H10 viruses. Among the North American lineage viruses, antisera against rt/NJ/01, mal/MN/07, and rt/NJ/08 neutralized 20, 19, and 17 H10 viruses, respectively. Antisera raised against the Eurasian lineage viruses dk/HK/79 and mal/PT/09 both neutralized all 20 viruses, while sera against JX/13 neutralized 18 of 20 viruses and mal/SW/02 antisera inhibited 15 of 20 viruses (Table 4). Thus, based on these assays, seven viruses induced a broadly cross-reactive H10 antibody response and could potentially be used as vaccine candidates.

TABLE 2 Unique amino acid constellations in the HAs of H10 viruses that display pronounced long-chain α 2,6-linked sialic acid binding

Virus	Amino acid at position 137 (H3 numbering)	Unique amino acid(s) within or in close proximity to receptor-binding site		Region in HA receptor-binding site
		H10 numbering ^a	H3 numbering	
ql/IT/65	137R	120P	129P	130 loop
		130S	138S	130 loop
		149G	157G	150 loop
		211I	217I	220 loop
ck/NSW/10	137S	129S	137S	130 loop
		D187N	D193N	190 helix
rt/NJ/01	137R	129R	137R	130 loop
rt/NJ/08	137R	230V	236V	Proximal to 220 loop
		185R	191R	190 helix
mk/SW/84	137K	112T	121T	Proximal to 130 loop
		117A	126A	Proximal to 130 loop
		143V	151V	Proximal to 150 loop
		150D	158D	150 loop
sl/GM/14	137K	212R	218R	220 loop
		113N	122N	Proximal to 130 loop
		119E	128E	130 loop
		165A	171A	Proximal to 150 loop
		204K	210K	Proximal to 220 loop

^aH10 numbering was derived from the HA sequence after removal of the signal peptide. H10 numbering was converted to H3 numbering as described previously (57).

DISCUSSION

Given the recent zoonotic and interspecies transmission of H10 influenza viruses, we undertook studies to characterize the subtype and to select candidate H10 vaccine strains. The H10 viruses in our study universally replicated in the upper respiratory tract of ferrets, with variable replication in the lower respiratory tract and variable, strain-specific weight loss. Characterization of the receptor-binding specificity demonstrated that H10 viruses have an α 2,3-linked sialic acid binding preference; however, several isolates also exhibited binding to long-chain α 2,6-linked sialic acids, and some H10 isolates had neuraminidase activity that was comparable to that of human influenza viruses. To identify potential vaccine candidates, we evaluated the cross-HI and neutralizing antibody response and found that 7 antisera cross-reacted with ≥ 15 of 20 H10 viruses.

We evaluated the ability of the H10 viruses to cause weight loss in ferrets because human infections with H10 viruses have resulted in a range of disease severity from mild illness to death. None of the viruses caused severe disease, and only three viruses caused $>10\%$ weight loss. Of interest, mk/SW/84 which caused $\sim 30\%$ mortality and $\sim 100\%$ morbidity in mink (11), was not lethal for ferrets despite causing substantial weight loss. Furthermore, infection of ferrets with JX/13 and sl/GM/14, which have caused lethal infections in humans and seals, did not result in pronounced weight loss or clinical disease. Thus, secondary bacterial infection or comorbidities may have contributed to severe disease in humans and other mammals (32) or there may be virulence factors that are not detected in the ferret model.

The majority of viruses grew to high titers in nasal turbinates but exhibited variable titers in the lungs, between 10^2 and 10^6 TCID₅₀/g. Three isolates (bldk/NB/10, ql/IT/65, and ck/GM/49), grew poorly in both nasal turbinates and lungs, while ck/NSW/10 and mk/SW/84 both grew to high titers in these tissues. Both JX/13 and sl/GM/14 grew to high titers in nasal turbinates, but JX/13 grew poorly in the lungs, while sl/GM/14 exhibited moderate replication. Importantly, avian viruses, such as rt/NJ/01, rt/NJ/08, and mal/PT/09, with no history of transmission to mammals, replicated to high titers in nasal turbinates and moderate (i.e., 10^4 TCID₅₀/g) to high (i.e., 10^5 to 10^6 TCID₅₀/g) titers

FIG 6 Neuraminidase activity of H10 influenza viruses. The NA activity of H10 influenza viruses was assayed using an NA XTD kit (Roche). Shown are NA activities of H10 viruses from the North American (A) and Eurasian (B) lineages. NA assays were run twice independently, each in triplicate, and the results of a representative assay are displayed. Three human influenza viruses [A/California/07/2009 (H1N1), A/Perth/16/2009 (H3N2), and A/Japan/305/1957 (H2N2)] were included to determine the range of NA activity exhibited by human viruses. For clarity, the viruses were divided by lineage, and the findings for the control viruses are presented in both panels. The error bars indicate standard errors of the means.

in the lungs. To determine if the H10 viruses carried mutations or gene constellations that are associated with replication in mammals, we analyzed the available PB2 gene sequences for known mammalian-adaptive mutations (33). As shown in Table 1 and as previously described (8, 21), only 3 viruses carried known mutations, and the JX/13 virus had internal protein gene segments donated by H9N2 viruses (21). Thus, there was no association between known internal protein gene mutations and replication in ferrets. Collectively, our findings suggest that many H10 viruses replicate in mammals without prior adaptation. Importantly, we previously evaluated a range of other avian influenza viruses, including H2, H5, and H6 subtypes, and found that many avian influenza viruses can also replicate in mice and ferrets (34–36).

In previous studies, three H10 viruses were evaluated in ferrets. These isolates were tk/MN/79, A/teal/Egypt/12908-NAMRU3/2005 (H10N1), and A/harbor seal/Germany/PV20762_TS/2014 (H10N7) (32, 37, 38). In studies with tk/MN/79, ferrets shed virus for 7 days, with a peak titer of $10^{7.3}$ 50% egg infective doses (EID₅₀)/ml following intranasal inoculation (38). Our findings are consistent with this report, as we observed titers of

TABLE 3 Cross-HI titers of H10 ferret antisera

Virus	Antiserum titer ^a																		
	tk/MN/79	rt/NJ/01	nshov/WA/06	mal/MN/07	rt/NJ/08	sb/DE/09	npin/AK/09	bidk/NB/10	ck/NSW/10	ck/GM/49	qI/IT/65	dk/HK/79	mk/SW/84	mdk/SG/93	mal/SW/02	wig/NL/07	mal/PT/09	dk/TH/09	JX/13
tk/MN/79	80	160	320	640	640	80	<10	320	<10	<10	80	640	80	<10	640	160	1,280	160	320
rt/NJ/01	<10	160	<10	640	640	40	<10	160	<10	<10	80	640	40	40	320	160	640	80	160
nshov/WA/06	<10	80	160	640	320	40	<10	160	<10	<10	80	640	40	40	320	80	640	80	640
mal/MN/07	<10	80	160	1,280	640	40	<10	320	<10	<10	160	2,560	80	<10	1,280	320	1,280	80	640
rt/NJ/08	<10	160	160	1,280	1,280	80	<10	160	<10	<10	160	1,280	80	<10	1,280	320	1,280	160	640
sb/DE/09	<10	160	<10	1,280	1,280	160	<10	160	<10	<10	80	2,560	40	<10	2,560	320	1,280	80	1,280
npin/AK/09	<10	80	40	640	640	40	320	320	<10	<10	80	1,280	80	<10	1,280	320	1,280	160	320
bidk/NB/10	<10	320	320	1,280	1,280	160	<10	320	<10	<10	160	2,560	160	80	1,280	640	2,560	320	640
ck/NSW/10	<10	40	<10	80	80	20	<10	80	320	<10	40	320	<10	<10	160	160	320	<10	40
ck/GM/49	160	160	320	640	320	80	<10	640	<10	160	160	640	320	80	640	160	640	320	160
qI/IT/65	<10	80	<10	160	80	20	<10	160	<10	<10	320	160	40	<10	160	160	320	40	<10
dk/HK/79	<10	80	<10	1,280	1,280	40	<10	320	<10	<10	80	2,560	<10	<10	1,280	160	2,560	160	640
mk/SW/84	<10	160	<10	320	<10	40	<10	160	<10	<10	80	320	320	40	160	80	320	80	<10
mdk/SG/93	<10	80	<10	80	40	20	<10	80	<10	<10	40	80	40	160	80	80	80	40	20
wig/NL/07	80	160	320	1,280	1,280	160	<10	320	<10	<10	80	2,560	80	80	640	2,560	2,560	160	640
mal/PT/09	<10	80	<10	1,280	640	20	<10	160	<10	<10	80	2,560	80	80	1,280	640	1,280	160	640
dk/TH/09	<10	160	160	640	80	40	<10	320	<10	<10	40	640	<10	<10	320	160	640	320	<10
JX/13	<10	1,280	320	640	320	320	<10	1,280	<10	160	320	640	320	160	640	320	640	320	640
sI/GM/14	<10	160	320	320	<10	80	640	320	320	40	80	640	320	160	320	640	640	320	<10
Total no. of viruses inhibited	3	20	11	17	14	16	2	20	2	3	16	16	13	10	19	18	19	17	14

^aInhibition (boldface) was defined as ≤ 4 -fold decrease relative to homologous titers (shaded). The lower limit of detection was 10.

TABLE 4 Neutralization titers of select H10 ferret antisera against H10 influenza viruses

Virus	Antiserum titer ^a									
	rt/NJ/01	mal/MN/07	rt/NJ/08	sb/DE/09	dk/HK/79	mal/SW/02	wig/NL/07	mal/PT/09	dk/TH/09	JX/13
tk/MN/79	403	403	508	254	806	508	453	806	202	320
rt/NJ/01	806	320	806	453	806	508	905	905	101	320
nshov/WA/06	403	403	127	113	254	226	202	453	57	160
mal/MN/07	453	508	508	403	806	508	453	905	127	254
rt/NJ/08	508	320	905	320	905	508	453	508	101	320
sb/DE/09	1,280	905	>2,560	1,280	1,810	1,280	2,032	1,016	226	1,613
npin/AK/09	806	453	640	254	905	1,016	403	640	254	453
bldk/NB/10	640	508	640	320	1,575	1,016	905	806	202	403
ck/NSW/10	403	226	226	160	508	254	202	403	40	202
ck/GM/49	254	905	127	127	806	508	508	226	127	202
ql/IT/65	320	113	320	101	320	226	254	254	63	113
dk/HK/79	403	320	403	226	403	453	453	508	226	403
mk/SW/84	403	226	226	101	320	226	226	320	80	113
mdk/SG/93	226	226	226	80	226	63	226	254	20	320
mal/SW/02	806	640	640	254	1,280	1,613	1,810	3,225	806	905
wig/NL/07	1,280	905	1,016	320	1,280	1,016	2,560	1,810	508	640
mal/PT/09	508	403	508	403	508	806	640	905	160	254
dk/TH/09	453	508	508	254	1,280	905	453	1,613	640	403
JX/13	508	403	202	320	226	403	453	226	80	508
sl/GM/14	508	403	403	160	1,016	806	1,613	640	127	254
Total no. of viruses inhibited	20	19	17	8	20	15	7	20	9	18

^aInhibition (boldface) was defined as ≤ 4 -fold decrease relative to homologous titers (shaded). The lower limit of detection was 10.

tk/MN/79 in nasal turbinates between 10^6 and 10^8 TCID₅₀/g (Fig. 4A). Also in agreement with our findings, on day 4 postinfection of ferrets with A/teal/Egypt/12908-NAMRU3/2005 (H10N1), titers of 10^7 and 10^3 EID₅₀ were detected in nasal turbinates and lungs, respectively (37). For studies using A/harbor seal/Germany/PV20762_TS/2014 (H10N7), animals were inoculated intratracheally, and viral replication was evaluated throughout the respiratory tract (32). On days 3 and 7 postinfection, 1/3 and 0/3 animals, respectively, had virus in nasal turbinates, while virus was detected in the lungs of all 3 animals at each time point, with titers between $10^{5.3}$ and $10^{6.6}$ TCID₅₀/g on day 3 and $10^{1.6}$ to $10^{4.0}$ TCID₅₀/g on day 7. As we observed comparable titers in the lungs with the closely related sl/GM/14 virus but high titers (10^6 to 10^7 TCID₅₀/g) in nasal turbinates on both days 3 and 5, it is likely that this difference can be attributed to the different routes (i.e., intratracheal versus intranasal) of inoculation (32).

It is important to note that two closely related H10 viruses have been evaluated in squirrel monkeys and humans. When squirrel monkeys were infected with tk/MN/79, the animals displayed mild clinical illness with viral shedding from the nasopharynx for 4 days and a peak titer of 10^3 TCID₅₀/ml. In human trials, volunteers were intranasally inoculated with 10^6 EID₅₀ of A/turkey/Minnesota/3/1979 (H10N7). Viral shedding was detected in 6 of 15 volunteers (titers not reported) and mild clinical disease in 8 of 15 volunteers (38). The reports of mild illness and viral shedding with variable peak titers are consistent with our observations in ferrets. All 4 of the infected squirrel monkeys developed an HI titer of 32, while none of the volunteers in the clinical study had detectable HI antibody responses. While these studies were performed with closely related isolates, the absence of high HI titers following primary infection is consistent with our findings of low homologous HI titers.

As the receptor-binding specificity for $\alpha 2,6$ -linked sialic acids is a determinant of interspecies transmission (39–42), we next sought to determine the receptor-binding preferences of the H10 viruses. We chose $\alpha 2,3$ - and $\alpha 2,6$ -linked sialic acids of various chain lengths, as binding to $\alpha 2,6$ -linked sialic acids, particularly long-chain glycans, has been shown to be crucial for the transmission of pandemic influenza viruses (43, 44). In our analyses, we found that all the H10 viruses had an $\alpha 2,3$ -linked sialic acid binding preference, with some isolates also displaying binding to long-chain $\alpha 2,6$ -linked sialic acids. In particular, ql/IT/65 and ck/NSW/10 exhibited a high degree of binding to

long-chain α 2,6-linked sialic acids. While the receptor-binding preference of JX/13 has been the subject of extensive research, ours is the first report to characterize the binding preferences of a panel of diverse H10 isolates. In previous studies, A/green wing teal/Texas/Y171/2006 (H10N7) was shown to have a strong preference for α 2,3-linked sialic acids, with no binding to α 2,6-linked sialic acids (45), while A/mallard/Sweden/51/2002 (H10N2) was shown to display a α 2,3-linked sialic acid binding preference with some binding to α 2,6-linked sialic acids (46). Our findings that H10 isolates displayed a range of binding profiles to α 2,6-linked sialic acids reconcile these conflicting reports.

Consistent with the findings of many research groups (29, 45, 47–49), we found that JX/13 exhibits a strong preference for α 2,3-linked sialic acids with minimal binding to α 2,6-linked sialic acids. Based on the structure of the HAs from A/mallard/Sweden/51/2002 (H10N2) and JX/13, Vachieri et al. postulated that the mutation K137R in JX/13 may further enhance binding to α 2,6-linked sialic acids (46). Subsequent crystal structure and binding studies with the HA from JX/13 refuted this hypothesis, as the JX/13 HA exhibited very little or no binding to α 2,6-linked sialic acids (48). Combined analysis of the amino acid residues at position 137 and the glycan-binding profiles of the panel of H10 viruses (Table 2 and Fig. 5) does not clearly agree with either of these studies. The isolates q1/IT/65, rt/NJ/01, and rt/NJ/08 have arginine at position 137 (Table 2) and exhibit long-chain α 2,6-linked sialic acid binding; however, these viruses also have additional substitutions (i.e., 138S, 185R, or 230V) in or proximal to the receptor binding site. Collectively, these findings indicate that multiple changes likely contribute to or influence the receptor-binding preference of the H10 HA.

Comparison of the HA sequences for q1/IT/65 and ck/NSW/10 to those of the other viruses in the panel revealed unique amino acid constellations in key HA receptor-binding site domains of the viruses (Table 2). Mutations in the 150 loop, specifically K158A and D193T, in combination with Q226L and G228S in the 220 loop, have been shown to switch the receptor-binding preference of H10 viruses (29), while the combination of Q226L and G228S alone did not alter H10 binding (49). Although none of the viruses in our study carried these specific mutations, ck/NSW/10 had the mutations D193N and 137S, and this combination may promote binding to α 2,6-linked sialic acids without a loss of α 2,3-linked sialic acid binding. We will evaluate the contributions of these mutations to the binding of α 2,6-linked sialic acids in the future using reverse-genetics techniques.

The NA activity of H10 viruses was characterized because it has been shown to enhance interspecies transmission of influenza viruses (30, 31, 50) and can influence the replication of live-attenuated vaccine viruses (51, 52). For comparison, we also evaluated the NA activities of representative human H1, H2, and H3 viruses (Fig. 6). From the panel of H10 viruses, only 3 isolates exhibited activity that was comparable to that of human viruses, and 6 isolates had elevated activity. Importantly, ck/NSW/10, which transmitted to poultry workers, was one of the three viruses with activity that was comparable to that of human isolates. This virus also replicated efficiently in the ferret respiratory tract and displayed pronounced binding to α 2,6-linked sialic acids. These findings support the observed transmission to humans (19), and future studies evaluating respiratory transmission of the virus are warranted. While the ck/NSW/10 virus exhibits evidence of adaptation to mammals, it should also be noted that 11 of the 20 H10 strains had low NA activity, and this observation suggests that the balance of HA and NA activities may be a limiting factor in interspecies transmission of H10 viruses. Furthermore, as NA activity has been shown to influence the replication of live-attenuated vaccines (52, 53) and the H10 viruses that induced a broad cross-neutralizing response displayed a range of NA activities, it may be necessary to introduce mutations or to generate reassortant viruses with different neuraminidases from the H10 subtype to generate vaccine candidates that exhibit optimal growth properties in embryonated chicken eggs.

To select an H10 vaccine candidate, we generated antisera against the panel of H10 viruses and performed cross-HI and neutralization assays. We found that antisera

against rt/NJ/01, mal/MN/07, rt/NJ/08, dk/HK/79, mal/SW/02, mal/PT/09, and JX/13 cross-reacted with a wide range of H10 viruses, as determined by both HI and neutralization assays. Our goal was to select a virus that could (i) induce cross-reactive antibodies that neutralized a broad range of H10 viruses and (ii) cause weight loss and replicate throughout the respiratory tract of ferrets to allow evaluation of vaccine efficacy. Using these criteria, while all the viruses with broad cross-reactivity replicated efficiently in the upper respiratory tract, mal/SW/02 and JX/13 did not grow to high titers in the lung, precluding their selection as vaccine strains. Of the remaining strains, rt/NJ/01, mal/MN/07, rt/NJ/08, and dk/HK/79 did not cause pronounced weight loss over several days. In contrast, mal/PT/09 replicated to high titers in both the lungs and nasal turbinates and caused weight loss in ferrets despite exclusive α 2,3-linked sialic acid binding (Fig. 3 to 5). Furthermore, in the cross-HI assay (Table 3), relative to the homologous titers, antisera raised against mal/PT/09 and JX/13 had a <4 -fold decrease when evaluated against JX/13 and mal/PT/09, respectively, indicating the viruses are antigenically similar. When the mal/PT/09 and sl/GM/14 sera were evaluated similarly, the mal/PT/09 sera cross-reacted with the sl/GM/14 virus, while the sl/GM/14 antisera failed to cross-react with the mal/PT/09 virus. These findings suggest that if used as a vaccine, mal/PT/09 would induce a response capable of recognizing the two most recent isolates in our panel. Thus, we conclude that mal/PT/09 represents a suitable H10 virus for further development.

In summary, we characterized a large number of H10 viruses and found that both avian and mammalian H10 isolates exhibited biological properties that could enable interspecies transmission, suggesting that H10 viruses will continue to cross the species barrier and cause sporadic outbreaks in mammals. Furthermore, our data are consistent with the epidemiological evidence showing that H10 viruses have a propensity to infect humans and may be capable of onward transmission. Thus, the development of an H10 vaccine is warranted, and we identified mal/PT/09 as a suitable vaccine strain. Our analysis also reconciles differing reports on the receptor-binding preference of avian H10 viruses and suggests that some H10 viruses carry previously unidentified combinations of amino acid changes that may permit binding to α 2,6-linked sialic acids while maintaining an α 2,3-linked sialic acid receptor-binding preference. Collectively, these findings underscore the need to develop an H10 pandemic vaccine and indicate that future study of H10 viruses will enhance our understanding of interspecies transmission.

MATERIALS AND METHODS

Selection and culture of virus strains. Phylogenetic analysis was performed on all H10 HA sequences from the NCBI influenza virus and GISAID databases. Incomplete or mixed H10 HA sequences were removed from the analysis. Evolutionary history and phylogenetic trees were generated using the maximum-likelihood method with a Tamura-Nei substitution model in MEGA7 software (54). Viruses spanning the entire H10 HA tree that encompassed all 9 NA subtypes were selected. The list of viruses is provided in Table 1 and Fig. 1. Viruses were kindly provided by or obtained from the NIH Laboratory of Infectious Diseases collection [A/turkey/Minnesota/5/1979 (H10N7)]; D. Stallknecht, University of Georgia [A/ruddy turnstone/New Jersey/471613/2001 (H10N7)]; H. Ip, U.S. Geological Survey, National Wildlife Health Center [A/northern shoveler/Washington/44249-675/2006 (H10N2) and A/American black duck/New Brunswick/00477/2010 (H10N6)]; M. Osterholm and C. Cardona, University of Minnesota [A/mallard/Minnesota/SG057/2007 (H10N7) and A/ruddy turnstone/New Jersey/SG-498/2008 (H10N7)]; R. Webby, St. Jude Children's Research Hospital [A/shorebird/Delaware Bay/139/2009 (H10N7) and A/mink/Sweden/E12665/1984 (H10N4)]; J. Runstadler, Massachusetts Institute of Technology [A/northern pintail/Interior Alaska/9BM5376R0/2009 (H10N7)]; A. Hurt, WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia [A/chicken/New South Wales/D809/2010 (H10N7)]; BEI Resources [A/chicken/Germany/N/1949 (H10N7) and A/quail/Italy/1117/1965 (H10N8)]; H. Kida, Hokkaido University, Japan [A/duck/Hong Kong/562/1979 (H10N9)]; T. Huangfu, Agri-Food and Veterinary Authority of Singapore, Singapore [A/mandarin duck/Singapore/805/F-72/7/1993 (H10N5)]; J. Waldenstrom, Linnaeus University, Sweden [A/mallard/Sweden/770/2002 (H10N6) and A/mallard/Portugal/79906/2009 (H10N7)]; R. A. M. Fouchier, Erasmus Medical Center, Netherlands [A/Eurasian wigeon/Netherlands/4/2007 (H10N1) and A/seal/Germany/EMC-1/2014 (H10N7)]; N. Bunpaong, Center of Emerging and Reemerging Infectious Diseases in Animals, Bangkok, Thailand [A/duck/Thailand/LM-CU4775/2009 (H10N3)]; and Y. Shu, Chinese Center for Disease Control, China [A/Jiangxi-Donghui/346/2013 (H10N8)].

All viruses except A/seal/Germany/EMC-1/2014 (H10N7) were cultured in specific-pathogen-free 10- to 11-day-old embryonated chicken eggs (Charles River, Franklin, CT) at 35°C. A/seal/Germany/EMC-1/

2014 (H10N7) was cultured and all virus stocks were titrated on Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) at 37°C. Virus titers were calculated using the Reed and Muench method (55) and expressed as the TCID₅₀.

Viral replication, weight loss, and generation of antisera in ferrets. Twenty-four-week-old ferrets ($n = 8$ or 9/virus; Triple F Farms) were screened by hemagglutination inhibition assay to be seronegative for circulating H1N1 and H3N2 influenza viruses. The ferrets were inoculated intranasally with 1×10^6 TCID₅₀ of each H10 virus in a 1-ml volume under 5% inhalational isoflurane. The animals were weighed daily for 14 days. On days 3 and 5 postinfection (p.i.), 3 animals per group were humanely euthanized, and brain, nasal turbinate, and lung tissues were collected, flash frozen on dry ice, and stored at -80°C . On day 28 p.i., terminal blood collections were performed on the remaining ferrets, and the blood was processed to isolate sera for serological assays.

To determine levels of replicating virus in each tissue, frozen tissues were thawed, weighed, and homogenized at a ratio of 10% (wt/vol) in Leibovitz-15 (L15) medium supplemented with antibiotic-antimycotic (200 units/ml penicillin, 200 $\mu\text{g/ml}$ streptomycin, and 0.5 $\mu\text{g/ml}$ amphotericin B) (Life Technologies). Tissue homogenates were clarified by centrifugation at $1,000 \times g$ for 10 min and were titrated in 24- and 96-well tissue culture plates of MDCK cells containing minimal essential medium (MEM) supplemented with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated (TPCK)-trypsin (Worthington Biochemical, Lakewood, NJ). The viral titers were expressed as log₁₀ TCID₅₀ per gram of tissue.

Glycan-binding preference of H10 influenza viruses. The receptor specificities of the 20 H10 viruses were evaluated using a panel of glycans as described previously (44) with minor modifications. Briefly, streptavidin-coated 384-well plates were incubated with 2.4 μM biotinylated glycans overnight at 4°C. The panel of glycans included 3'SLN, 3'SLN-LN, 3'SLN-LN-LN, 6'SLN, 6'SLN-LN, and 6'SLN-LN-LN. LN corresponds to lactosamine (Gal β 1-4GlcNAc), and 3'SLN and 6'SLN, respectively, correspond to Neu5Ac α 2-3 and Neu5Ac α 2-6 linked to LN. Each additional LN in the nomenclature corresponds to an increase in the length of the glycan. These glycans were specifically chosen because they represent both short- and long-chain variants. The 3'SLN-LN and 6'SLN-LN glycans were obtained from the Consortium of Functional Glycomics (<http://www.functionalglycomics.org>), and the remaining glycans were purchased from Sussex Research (Ottawa, ON, Canada). Each H10 virus was diluted to 128 hemagglutinating units (HAU)/50 μl and then further diluted 1:5 in phosphate-buffered saline (PBS). The diluted virus (50 $\mu\text{l/well}$) was then incubated on the glycan-coated plates overnight at 4°C. The plates were washed four times with PBST (1 $\times$ PBS plus 0.05% Tween 20) and blocked with Superblock T20 (ThermoScientific) for 2 h at 4°C. After rinsing 4 times with PBST, the plates were incubated with a 1/10,000 dilution of anti-H10 goat hyperimmune serum [BEI Resources; Polyclonal anti-influenza virus H10 A/chicken/Germany/N/1949 (H10N7); NR-3153] in PBST-SB20 (equal parts PBST and Superblock T20) for 2 h at 4°C. Following an additional rinse step, the plates were incubated with donkey anti-goat horseradish peroxidase (HRP)-labeled polyclonal serum (1/50,000 in PBST-SB20; Rockland Laboratories, Limerick, PA) for 1 h at 4°C. After incubation, the plates were washed 4 times with PBST and twice with PBS. HRP activity was quantified using an Amplex Red peroxidase assay (ThermoScientific) according to the manufacturer's instructions and measured on a fluorescent-plate reader (SpectraMax; Molecular Devices, Sunnyvale, CA).

As control viruses, the QG and LS variants of A/Japan/305/1957 (H2N2) ca vaccine, which preferentially bind α 2,3- and α 2,6-linked sialic acids, respectively, were included in the assay (28, 53). For detection of H2 virus binding, wells containing H2 viruses were incubated with a 1/10,000 dilution of anti-H2 goat hyperimmune serum [BEI Resources; polyclonal anti-influenza virus H2 HA; A/Singapore/1/1957 (H2N2); NR-4523] and were rinsed and developed as described above. Negative controls included no-glycan (i.e., PBS-only-coated) wells, glycan-coated wells without virus, and glycan-coated wells incubated with virus and secondary antibody (i.e., no primary antibody). The last control was found to have the highest background signal, and the mean value of these "no-primary-antibody" controls for each virus at a given glycan length was subtracted from the binding signal. To control for differences in binding of the polyclonal hyperimmune sera to each H10 virus, the binding signal was expressed as a percentage of the highest binding signal detected for a given virus. The glycan-binding assays were performed twice in triplicate.

Sequencing of the H10 HA genes for tk/MN/79 and mdk/SG/93. To sequence the HA genes for tk/MN/79 and mdk/SG/93, viral RNA was extracted from undiluted virus stock using a QIAamp viral RNA kit (Qiagen, Hilden, Germany). Reverse transcription was performed using Superscript IV reverse transcriptase (Life Technologies, Waltham, MA) and Uni-12 primer (Integrated DNA Technologies, Coralville, IA). The HA gene segment was subsequently amplified using GoTaq DNA polymerase (Promega, Madison, WI) and segment-specific primers (56). PCR products were purified after gel electrophoresis using a QIAquick gel extraction kit (Qiagen) and were subjected to sequencing using a BigDye Terminator v3.1 cycle-sequencing kit on a 3730 DNA analyzer (Applied Biosystems, Foster City, CA).

H10 HA and PB2 sequence alignment. To identify mutations that might be associated with receptor-binding specificity or mammalian adaptation, the published HA sequences for 18 viruses corresponding to NCBI accession numbers CY144820, CY076269, CY034636, CY145565, CY137794, CY135567, CY138933, CY014671, CY087832.1, AB292781, M21646, CY183613, CY077048, CY116608, and CY062596 and GISAID accession numbers EPI397852, EPI497477, and EPI544351 were analyzed, along with laboratory-generated sequences for tk/MN/79 and mdk/SG/93. Available PB2 gene sequences were downloaded, and the HA and PB2 sequences were aligned using Muscle in MacVector (Apex, NC). For analysis of HA sequences, the signal peptide was deleted from the HA, and unique mutations identified in the H10

sequences were converted to H3 numbering (57). For analysis of the PB2 sequences, all available sequences were analyzed for previously described mutations (33).

Neuraminidase activity. The neuraminidase activity of the H10 viruses was assayed using an NA-XTD influenza neuraminidase kit (ThermoFisher). Virus stocks were diluted to a concentration of 2×10^6 TCID₅₀/ml, and serial 2-fold dilutions were prepared starting with 100 μ l of diluted virus. The virus was diluted in NA-XTD buffer, and the neuraminidase activity was measured according to the manufacturer's protocol. Three additional wild-type human influenza viruses [A/California/07/2009 (H1N1), A/Perth/16/2009 (H3N2), and A/Japan/305/1957 (H2N2)] and uninfected allantoic fluid were included as controls. NA activity was assayed in triplicate for each virus, and the NA activity was assayed twice, independently.

Evaluation of cross-reactive antibody titers against heterologous and homologous H10 viruses. (i) Hemagglutination inhibition. Due to low HI titers, day 28 ferret sera were concentrated 10-fold using Amicon Ultra-15 filter units (100,000-kDa molecular mass limit; Millipore, Billerica, MA). Concentrated sera were treated with receptor-destroying enzyme (RDE) (Denken Seiken, Tokyo, Japan) and tested for HI according to standard protocols (58) using turkey red blood cells (0.5% suspension in normal saline). Homologous HI titers were recorded as the reciprocal dilution that completely inhibited hemagglutination of 4 HA units of each H10 virus. Ferret antisera generated against each virus were evaluated for cross-inhibition of all 20 H10 viruses. A cross-reactive response was defined as a ≤ 4 -fold decrease between the homologous HI titer and the titer against a heterologous H10 virus.

(ii) Neutralization. After screening for cross-reactive HI titers, 10 antisera were further evaluated for their breadth of neutralizing antibody titers as previously described (35). For these assays, serial 2-fold dilutions of unconcentrated heat-inactivated sera were prepared at a starting dilution of 1:20. The diluted sera were incubated for 60 min at room temperature with 100 TCID₅₀ of each virus. The residual infectivity was then evaluated by overlaying the serum-virus mixture on MDCK cells grown in 96-well plates (4 replicates per dilution of sera). The plates were incubated for 4 days and scored for cytopathic effect (CPE). The antibody titers were expressed as the reciprocal of the geometric mean titer that completely neutralized virus infection, and a ≤ 4 -fold decrease between the homologous titer and the titer against a heterologous H10 virus was considered cross-reactive or cross-protective.

Biocontainment and animal protocols. For most of the viruses evaluated, virus culture and ferret studies were performed under biosafety level 2 conditions. Experiments with A/mandarin duck/Singapore/805/F-72/7/1993 (H10N5), A/Jiangxi-Donghui/346/2013 (H10N8), and A/seal/Germany/EMC-1/2014 (H10N7) were conducted in enhanced biosafety level 3 laboratories at the NIH. All animal studies and procedures were conducted in compliance with protocols approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee.

Accession number(s). Laboratory-generated sequences for tk/MN/79 and mdk/SG/93 were deposited in the NCBI database under accession numbers [MF613654](#) and [MF613655](#), respectively.

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