Pathogenesis, transmissibility, and ocular tropism of a highly pathogenic avian influenza A (H7N3) virus associated with human conjunctivitis.

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Abstract
H7 subtype influenza A viruses, responsible for numerous outbreaks in land-based poultry in Europe and the Americas, have caused over 100 cases of confirmed or presumed human infection over the last decade. The emergence of a highly pathogenic avian influenza H7N3 virus in poultry throughout the state of Jalisco, Mexico, resulting in two cases of human infection, prompted us to examine the virulence of this virus [A/Mexico/InDRE7218/2012 (MX/7218)] and related avian H7 subtype viruses in mouse and ferret models. Several high and low pathogenicity H7N3 and H7N9 viruses replicated efficiently in the respiratory tract of mice without prior adaptation following intranasal inoculation, but only MX/7218 virus caused lethal disease in this species. H7N3 and H7N9 viruses were also detected in the mouse eye following ocular inoculation. Virus from both H7N3 and H7N9 subtypes replicated efficiently in the upper and lower respiratory tract of ferrets, however, only MX/7218 virus infection caused clinical signs and symptoms and was capable of transmission to naïve ferrets in a direct contact model. Similar to other highly pathogenic H7 viruses, MX/7218 replicated to high titers in human bronchial epithelial cells, yet downregulated numerous genes related to NF-κB-mediated signaling transduction. These findings indicate that the recently isolated North American lineage H7 subtype virus associated with human conjunctivitis is capable of causing severe disease in mice and spreading to naïve contact ferrets, while concurrently retaining the ability to replicate within ocular tissue allowing the eye to serve as a portal of entry.
Introduction

Avian influenza A (H7) viruses have been responsible for numerous zoonotic transmissions from poultry to humans since 2002 (3). The largest H7 outbreak in humans, resulting in over 80 cases with one fatality, occurred in The Netherlands in 2003, and was caused by a highly pathogenic avian influenza (HPAI) H7N7 virus that led to the culling of over 30 million birds (20, 30). While outbreaks of HPAI H7N3 viruses in poultry have previously occurred in Chile in 2002, British Columbia, Canada in 2004, and Saskatchewan, Canada in 2007 (11, 23, 52, 54), a recent outbreak of HPAI H7N3 virus in Jalisco, Mexico, necessitating the culling of over 20 million birds, represents the largest documented epornitic of HPAI virus in North America to date (13, 19). Two virologically confirmed human cases were detected following exposure to infected poultry, both presenting with conjunctivitis but without fever or respiratory symptoms (13). The scale of this poultry outbreak and its association with the first documented cases of human infection with an H7 virus in five years highlights the need for continued surveillance and study of this virus subtype.

North American lineage H7 avian influenza viruses generally exhibit reduced virulence in mammalian models compared with some equine H7N7 and Eurasian lineage H7 avian influenza viruses, which more closely resemble HPAI H5N1 viruses in their ability to cause systemic, lethal disease (1, 5, 25, 27). However, several North American low pathogenicity avian influenza (LPAI) H7N2 and HPAI H7N3 viruses are highly infectious in mice and capable of limited transmission among ferrets in direct contact (2, 5, 51). Unlike other virus subtypes, H7 influenza viruses of both lineages have been predominantly associated with ocular disease in humans, typically conjunctivitis (3, 30, 54). Most recently, a HPAI H7N3 virus isolated from a human conjunctivitis case during the 2004 poultry outbreaks in British Columbia, Canada was...
shown to replicate efficiently in numerous human respiratory and ocular cell types (9, 10). However, it was unknown if other North American H7N3 and H7N9 viruses shared these properties.

H7N3 viruses have been the cause of all highly pathogenic avian H7 subtype influenza virus outbreaks in North America. However, the isolation of numerous LPAI H7N3 and H7N9 viruses in North America during the past decade underscores the potential for the generation of pathogenic variants from these LPAI precursors and the risk of human exposure (19, 32). To better understand the ability of North American H7N3 viruses to cause respiratory and ocular disease in mammalian species, we examined the pathogenesis, transmissibility, and ocular tropism of a virus isolated from one of the HPAI H7N3 human infections detected in Jalisco, Mexico in 2012, A/Mexico/InDRE7218/12 (MX/7218). We found that this virus exhibited enhanced virulence in both mouse and ferret models compared with several phylogenetically related LPAI H7N3 and H7N9 isolates and closely mirrored previously studied HPAI H7 human isolates in the capacity for efficient replication and subtype-specific activation of host responses in human respiratory cells.
Materials and Methods

Viruses. Influenza A viruses of the H7 subtype used in this study are shown in Table 1. Virus stocks were propagated in the allantoic cavity of 10-day-old embryonated hen’s eggs at 37°C for 24 to 26 h (HPAI) or 35°C for 36-48 h (LPAI). Allantoic fluid pooled from multiple eggs was clarified by centrifugation and frozen in aliquots at -70°C. The 50% egg infectious dose (EID50) for each virus stock was calculated by the method of Reed and Muench following serial dilution in eggs (49). Viruses were additionally tested by standard plaque assay in Madin-Darby Canine Kidney (MDCK) cells (ATCC, Manassas, VA) for determination of PFU titer. All research with HPAI viruses was conducted under biosafety level 3 containment, including enhancements as outlined in Biosafety in Microbiological and Biomedical Laboratories (14).

Mouse experiments. Female BALB/c mice (Jackson Laboratories), 6 to 8 weeks of age, were anesthetized with 2,2,2-tribromoethanol in tert-amyl alcohol (Avertin; Sigma-Aldrich, St. Louis, MO). Mice were inoculated with 10^6 EID50 of virus by the intranasal (i.n.) route (50μl volume), or the ocular (i.o.) route (5μl volume) dropped onto the corneal surface of the right eye and massaged in with the eyelids following corneal scarification as described previously (8). Five to seven mice per virus were monitored daily for 14 days post-inoculation (p.i.) for morbidity, as measured by weight loss, and mortality for determination of a 50% lethal dose (LD50). Any mouse that lost greater than 25% of its pre-infection body weight was euthanized. Replication and systemic spread of each virus was determined by harvesting the right eye, nose, lung, and brain of mice (n=3) on days 3 and 6 p.i., and tissues were titrated in eggs as described previously (limit of detection, 10^{1.5} EID50/ml i.n. tissues, 10^{0.8} EID50/ml i.o. tissues) (34).

Ferret experiments. Male Fitch ferrets (Triple F Farms, Sayre, PA), 4 to 6 months of age and serologically negative by standard hemagglutination inhibition for currently circulating influenza
viruses, were used in this study. Ferrets were housed for the duration of each experiment in a
Duo-Flo Bioclean mobile unit (Lab Products Incorporated, Seaford, DE). Ferrets (n=3) were
inoculated i.n. with $10^6$ EID$_{50}$ of each indicated virus, observed daily for clinical signs and
symptoms of infection, with nasal washes collected on indicated days p.i. as previously described
(38). A serologically naïve ferret was placed in the same cage as an inoculated ferret 24 h p.i. for
assessment of virus transmission between ferrets in direct contact (37). An additional 3 ferrets
inoculated with $10^6$ EID$_{50}$ of each virus were euthanized day 3 p.i. for the assessment of virus
replication and systemic spread as previously described (38).

**Cell culture and viral replication.** The human bronchial epithelial cell line Calu-3 (ATCC)
grown on membrane inserts were cultured as previously described; these polarized cells possess
protease activity that can support human and avian influenza virus hemagglutinin cleavage
without the addition of exogenous TPCK-trypsin (56). Cells were grown to confluence in 6-well
plates for one week. Virus was added apically in serum-free medium to cells at a multiplicity of
infection (MOI) of 0.01 (replication kinetics) and incubated for 1 h before washing. Aliquots of
culture supernatant taken p.i. were immediately frozen at $-80^\circ$C until use. Medium with equal
amounts of virus-free normal allantoic fluid (10% total volume) was used as a mock control.
Samples collected for replication kinetics were titrated for the presence of infectious virus by
standard plaque assay in MDCK cells.

**Real-time RT-PCR.** RNA isolations were performed from clarified ferret lung tissue
homogenates with a QIAamp Viral RNA kit (Qiagen, Valencia, CA). Semiquantitative RT-PCR
was performed with primer sequences as described previously (36), with expression levels
normalized to Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and reported as fold
change compared with mock-infected animals. Calu-3 cells were propagated in 12-well plates
and infected with virus at an MOI of 2 as described above (Qiagen). Total RNA was extracted at 24 h p.i. RT² Profiler PCR array analysis (SABiosciences, Frederick, MD) for the human NF-κB signaling pathway was performed in triplicate with total RNA extracted at 24 h p.i. and analyzed per manufacturer's instructions.

**Statistical analysis.** Statistical significance (p<0.05) for murine and in vitro replication studies was determined by one-way ANOVA with a Bonferroni post-test. Statistical significance for ferret studies and PCR array analysis was determined using Student’s t test.

**Results**

**Pathogenicity of H7N3 and H7N9 influenza A viruses following intranasal inoculation in mice.** The BALB/c mouse model has been used extensively to study the pathogenesis of H7 influenza viruses administered by the i.n. route (5, 16, 17, 25). These studies have revealed that H7 viruses are highly infectious in mice without prior adaptation, with the capacity for systemic spread and lethal disease depending on the strain. We utilized this model to assess the virulence of the recently isolated HPAI H7N3 virus MX/7218, which was associated with human infection, and related LPAI and HPAI H7 subtype viruses with common phylogenetic ancestors (33) (Table 1). The HPAI H7N3 virus MX/7218 was isolated from a patient with conjunctivitis (13).

Isolates from Nebraska (LPAI H7N9) and Chile (LPAI and HPAI H7N3) were associated with outbreaks in domestic poultry flocks (45, 47, 52), whereas isolates from New Jersey and Arkansas (LPAI H7N3) were detected during surveillance activities (17, 48).

In agreement with previous studies which have reported lethality following inoculation with selected HPAI H7N3 viruses in mice (1, 25), MX/7218 virus-infected mice showed the greatest signs of illness following i.n. inoculation, resulting in >20% weight loss and an LD₅₀ of
10^5.5 EID_{50}. In contrast, the HPAI and LPAI H7N3 viruses from Chile in 2003 did not cause substantial morbidity and possessed an LD_{50} >10^7 EID_{50} (Figure 1A). Despite the disparity in morbidity between these viruses, the HPAI H7N3 viruses MX/7218 and ck/Chile/4322-2 replicated equally well in the lungs of mice, with titers of ≥10^6.5 EID_{50}/ml persisting through day 6 p.i. (Figure 1B-C). Virus was also detected to highest titer in the nose following HPAI H7N3 virus inoculation (>10^4.5 EID_{50}/ml) on day 3 p.i., with MX/7218-inoculated mice possessing nose titers at this time that were significantly higher than all other H7N3 and H7N9 viruses tested (p<0.05). Despite the lethal disease following high-dose MX/7218 virus inoculation, systemic spread to the brain was not detected on day 6 p.i. (data not shown) and mice showed no signs of neurologic symptoms.

Murine inoculation with LPAI H7N9 viruses from 2011 caused greater weight loss (13.3-17.0% on day 3 p.i.) compared with LPAI H7N3 viruses from 2006-8 (4.1-9.17% day 2-3 p.i.), but all mice recovered by the end of the observation period. All LPAI viruses examined replicated efficiently in the murine lung following i.n. inoculation, with mean lung titers ≥10^4.75 EID_{50}/ml and ≥10^5.2 EID_{50}/ml days 3 and 6 p.i., respectively (Figure 1D-H). In summary, while both HPAI H7N3 viruses MX/7218 and ck/Chile/4322-2 replicated to highest titers in the lung, only MX/7218 virus exhibited a lethal phenotype in mice at the doses tested. LPAI H7N3 and H7N9 viruses which exhibited reduced morbidity in this model nonetheless replicated efficiently in this species without the need for prior adaptation.

Pathogenicity of H7N3 and H7N9 influenza viruses following ocular inoculation in mice. H7 virus infection in humans frequently results in ocular and not respiratory disease (3). We previously established a mouse model of ocular inoculation which corroborated that H7 subtype
influenza viruses associated with human conjunctivitis during 2003-4 were capable of efficient replication in both eye and respiratory tract tissues following this route of inoculation (8). To assess if this property has been maintained among recently isolated North American lineage H7 viruses, mice were inoculated by the ocular (i.o.) route following corneal scarification with selected HPAI and LPAI H7 viruses (Table 2). Similar to prior H7 viruses associated with human disease, MX/7218 virus replicated efficiently in both eye and nose tissues following i.o. inoculation. The LPAI H7N9 virus gs/NE/11 was also capable of replication in both tissues, albeit with reduced titer and frequency. In contrast, the LPAI H7N3 virus rk/NJ/06 was not capable of efficient replication following this route of inoculation. Unlike i.n. inoculation, mice inoculated by the ocular route did not exhibit significant morbidity or mortality following i.o. inoculation with any virus tested (data not shown). These findings closely mirror the pattern of virus isolation in mice following i.o. inoculation with other H7 viruses (8), and indicate that recently isolated H7 influenza viruses have maintained the ability to mount a productive infection following exposure to ocular tissue in vivo.

Pathogenicity and transmission of H7N3 and H7N9 viruses in ferrets. Previous studies have shown that both LPAI and HPAI H7N3 viruses are capable of efficient replication throughout the respiratory tract of ferrets; however, H7N9 viruses have not been examined in this model (1, 2, 26, 51). Ferrets were inoculated i.n. with 10⁶ EID₅₀ and either observed for 14 days p.i. for clinical signs of infection or euthanized on day 3 p.i. for assessment of systemic spread. The HPAI H7N3 virus MX/7218 caused fever and moderate but transient weight loss, with all ferrets surviving the observation period (Table 3). In contrast, inoculation with the LPAI H7N9 virus gs/NE/11 did not cause clinical signs or symptoms of illness in this species. Respiratory signs,
including sneezing and nasal discharge, were not observed following inoculation with either virus (data not shown). MX/7218 and gs/NE/11 viruses replicated efficiently in the nasal turbinates, trachea, and lungs of ferrets, and elevated mRNA levels of several proinflammatory cytokines and chemokines in lung tissue day 3 p.i. were detected from both H7 virus-infected ferrets. Both viruses were also detected at comparable titers in the olfactory bulb and brain, however MX/7218 was detected at a higher frequency compared with gs/NE/11, which was only detected in these tissues in one of three ferrets (Table 3). Neither virus was detected in intestinal tissue day 3 p.i., although MX/7218 virus was detected in rectal swabs days 3-5 p.i. Virus was not detected in eye or conjunctival tissues day 3 p.i. with either virus (data not shown). These data indicate that, despite disparate presentations of clinical signs and symptoms following virus inoculation, both MX/7218 and gs/NE/11 were capable of efficient, high-titer replication throughout the respiratory tract in ferrets.

Selected North American and Eurasian lineage H7 viruses have demonstrated the ability for limited transmission in a ferret direct contact model (2, 51). To evaluate if recently isolated H7 viruses maintained this property, three ferrets were each inoculated i.n. with 10^6 EID_{50} of either the H7N3 or H7N9 virus, with a naïve ferret cohoused with each inoculated ferret 24 h p.i. (Figure 2). Criteria for efficient transmission included detection of virus in nasal washes and seroconversion of convalescent sera (≥4-fold rise in antibody titer from pre-infection sera) from contact ferrets. Both MX/7218 and gs/NE/11 viruses replicated efficiently in the upper respiratory tract of inoculated ferrets, however peak mean nasal wash virus titers were significantly higher following MX/7218 (10^{6.0} EID_{50}/ml day 3 p.i.) compared with gs/NE/11 virus (10^{5.0} EID_{50}/ml day 1 p.i.) virus infection (p<0.05). MX/7218 virus transmitted to three of three ferrets when placed in direct contact, as determined by detection of infectious virus and
seroconversion of all contact ferrets. In comparison, gs/NE/11 virus was not detected in nasal
washes of contact ferrets and seroconversion was not observed. These findings demonstrate that
the human isolate MX/7218, but not the genetically related LPAI virus gs/NE/11, was capable of
transmission between ferrets in direct contact.

Tropism of H7N3 and H7N9 viruses in human respiratory cells. Previous studies have
identified that epithelial and endothelial cells from multiple human organ systems support
replication of H7 influenza viruses associated with disease in humans (9, 10, 57). To determine if
differences in virus pathogenicity observed in mouse and ferret models were indicative of
different replicative abilities in human cells, we compared the ability of H7N3 and H7N9 viruses
to replicate in polarized human bronchial epithelial cells (Calu-3). The HPAI H7N3 virus
MX/7218 replicated to highest titer in Calu-3 cells among all viruses examined, reaching titers
>10^7 PFU/ml by 36 h p.i. Furthermore, MX/7218 virus replicated to significantly higher titers
compared with all other viruses examined over the 72 h time period (p<0.05). ck/Chile/4322-2,
ck/Chile/180-54, and rk/NJ/06 viruses replicated with comparable efficiency in Calu-3 cells,
reaching higher titers compared with the LPAI viruses ck/AR/08 and both H7N9 viruses at 24-36
h p.i. before reaching generally comparable titers by 72 h p.i. Despite replicating efficiently in
the mouse and ferret respiratory tracts, titers of H7N9 viruses examined achieved significantly
lower titers (<10^4 PFU/ml, p<0.05) in Calu-3 cells compared with all HPAI H7N3 viruses at this
time point. In summary, we found that the HPAI H7N3 human isolate MX/7218 replicated to
higher titers in human bronchial epithelial cells compared with LPAI H7N9 viruses, which
exhibited the lowest overall titers.
Downregulation of NF-κB signaling in human respiratory cells following HPAI H7N3 virus infection. We previously determined that, despite retaining the capacity for efficient replication, H7 influenza viruses elicit a weakened and delayed induction of proinflammatory cytokines and chemokines in human respiratory cells (9). Furthermore, compared with other virus subtypes capable of human infection, H7 viruses elicit a broad downregulation of genes related to the NF-κB signaling pathway in respiratory cells but not ocular cells following virus infection (10). We examined a panel of genes related to NF-κB signaling by real-time PCR array analysis in Calu-3 cells following infection with the human isolate MX/7218 to assess the ability of this HPAI H7N3 virus to elicit host responses. Genes related to the NF-κB signal transduction pathway that were significantly (p<0.05) upregulated or downregulated (>3-fold) in Calu-3 cells at 24 h p.i. are shown in Figure 4 as fold change compared with mock-infected cells. MX/7218 virus infection resulted in the significant upregulation of several genes, primarily cytokines such as IFNβ1 (notably associated with potent virus replication), IFNγ, IL-6, CSF2, LTA and TNF, all of which function as NF-κB responsive genes (Figure 4). However, despite the upregulation of these genes, levels of many of them were approximately 4- to 18-fold lower than what is typically observed following HPAI H5N1 virus infection in these cells (10). This is comparable to the HPAI H7N7 virus A/Netherlands/219/03 (NL/219) which was previously shown to elicit reduced levels of these genes in Calu-3 cells (as indicated with gray bars in Figure 4). The only exception was IL-10, which was detected at higher levels following HPAI H7N3 and H7N7 virus infection compared with H5N1 and H1N1 virus subtypes (10). Similar to the HPAI H7N7 virus NL/219, HPAI H7N3 virus infection in respiratory cells resulted in a broad downregulation of NF-κB-related genes, with several of these genes detected at lower levels compared with H5N1 virus infection (gray bars, Figure 4). These results indicate that infection with the HPAI H7N3
virus MX/7218 elicits a comparable host response to other HPAI H7 viruses in respiratory cells, in accord with the tropism of this virus subtype.

**Discussion**

Two conjunctivitis cases from the 2012 HPAI H7N3 virus outbreak in the state of Jalisco, Mexico represent the first documented human infections with H7 viruses in recent years (3). However, LPAI and HPAI H7 viruses have been routinely detected in poultry and wild birds throughout North America and in several countries in Europe and Asia during the last decade (19, 45). Given the potential for human exposure in poultry workers or other risk-groups that come into frequent contact with infected birds, it is critical to understand the public health threat posed by contemporary H7 viruses, especially those that have demonstrated a capacity for human infection. In this study, we used two mammalian models to compare the relative virulence of the human H7N3 isolate MX/7218 to several closely related HPAI and LPAI viruses isolated from various avian species. Despite generally similar replication profiles in respiratory tract tissues between all viruses tested, we observed enhanced virulence and transmission of MX/7218 virus in mouse and ferret models. Furthermore, MX/7218 virus replicated to highest titer in human bronchial epithelial cells and elicited a pattern of host responses following infection typical of other HPAI H7 viruses.

Lethality has been reported previously among some HPAI H7N3 and H7N7 virus isolates following murine inoculation (1, 5, 25). Equine H7N7 viruses from North America were also found to be highly pathogenic in mice, possessing generally similar levels of lethality as MX/7218 virus (27). However, with the exception of a HPAI H7N7 virus associated with a fatal case from The Netherlands in 2003, HPAI H7 viruses do not maintain this high level of virulence.
in the ferret model. Detection of virus at modest titers in the ferret olfactory bulb and brain, as shown here with MX/7218 virus, has also been reported following infection with several LPAI and HPAI H7 strains (5, 26). The ability of gs/NE/11 virus to achieve high-titer replication throughout the respiratory tract while displaying a nonpathogenic phenotype is consistent with other LPAI H7 North American strains which have caused only limited morbidity in this species.

Detection of gs/NE/11 virus in the olfactory bulb and brain of one ferret day 3 p.i. is likely a reflection of high titers of virus replication present in the proximal nasal cavity, and is in accord with the sporadic detection of seasonal influenza viruses in these tissues despite maintaining a low pathogenicity phenotype in this species (58). H7N3 and H7N9 virus-infected ferrets in this study did not demonstrate frequent sneezing or nasal discharge, similar to prior studies with North American H7 lineage viruses, which were found to cause limited morbidity in this species (2). Levels of proinflammatory cytokine and chemokine transcripts in the lungs were generally comparable between both viruses tested and are in agreement with the viral loads detected in this tissue day 3 p.i. (Table 3), demonstrating the ability of HPAI and LPAI H7 influenza viruses to elicit innate immune responses in the lower respiratory tract following intranasal inoculation (6, 36).

While multiple virus subtypes are capable of mounting a productive infection in the upper respiratory tract of ferrets following ocular inoculation, only selected H5 and H7 viruses possess this capacity in mice (4, 7, 8, 53). Peak titers of gs/NE/11 and MX/7218 viruses in ocular and respiratory tract tissue in mice inoculated by the ocular route were comparable in magnitude to previous H7 viruses examined in this model; the reduced morbidity of MX/7218 virus following i.o. compared with i.n. inoculation was also consistent with previous studies (8). In contrast, rk/NJ/06 virus did not efficiently use the eye as a portal of entry in mice; this virus was
isolated during routine surveillance and not associated with human infection or a poultry outbreak (Table 1) (17). Interestingly, MX/7218 virus was not detected in eyes, conjunctiva, or conjunctival washes collected from i.n.-inoculated ferrets, unlike Eurasian HPAI H7N7 viruses which have demonstrated the ability to replicate in these tissues following i.n. or i.o. inoculation (1, 4). Future research among viruses within the H7 subtype is needed to identify molecular properties associated with ocular tropism as this subtype continues to cause ocular disease following human infection.

The transmission of MX/7218 virus to naïve ferrets in the presence of direct contact is not the first time an H7 subtype virus associated with ocular and not respiratory disease in humans has demonstrated this capacity. Ferrets inoculated with an HPAI H7N3 virus, isolated from a human case in British Columbia, Canada in 2004, transmitted low levels of virus ($\leq 10^2$ EID$_{50}$/ml) detected in the nasal washes of two ferrets in direct contact with inoculated animals in the absence of seroconversion (2). A LPAI H7N3 mallard virus isolated in 2001 also transmitted to two of three ferrets (with positive virus isolation and seroconversion) (51). HPAI H7N7 and LPAI H7N2 viruses have further demonstrated the capacity for efficient transmission in the presence of direct contact following either i.n. or i.o. inoculation in ferrets (2, 4). Despite the capacity of these H7 subtype viruses to transmit to naïve ferrets in the presence of direct contact, they are not transmissible by respiratory droplets (2); it is likely that MX/7218 virus shares this phenotype, though further study is required. These findings are similar to limited reports of direct contact transmission of LPAI H9N2 viruses but in contrast with wild-type HPAI H5N1 viruses, which have not demonstrated the ability for direct contact transmissibility (37, 55). While the small sample size of ferret transmission pairs used in this study precludes a rigorous statistical analysis of virus transmissibility (39), the detection of numerous H7N2, H7N3, and H7N7 strains
associated with human infection which possess a transmissible phenotype, inclusive of both North American and Eurasian lineages, illustrates the pandemic potential and public health threat posed by this virus subtype. While the presence or absence of a multibasic HA cleavage site is likely to influence mammalian replication capacity and in turn virus transmissibility, further examination of the receptor-binding properties of MX/7218 virus and molecular comparison of MX/7218 and gs/NE/11 viruses may provide further insight into these properties.

Human epithelial lung cells are permissive to multiple subtypes of influenza virus, with HPAI and LPAI H7 viruses demonstrating similar levels of infectivity in this cell type (9, 56). MX/7218 virus replicated most efficiently in Calu-3 cells in this study, reaching similar peak mean titers in this cell type as prior HPAI H7N3 and H7N7 viruses associated with human conjunctivitis; all of these viruses possess a glutamic acid at position 627 in PB2 (9). Despite the close HA gene similarity between the MX/7218 virus and the LPAI H7N9 viruses from 2011 (33), peak viral titers of H7N9 viruses were >3 logs lower than the human isolate, likely attributable to the presence of a multibasic cleavage site motif in MX/7218 virus. Interestingly, MX/7218 virus elicited a similar pattern of induction of NF-κB-related genes as the HPAI H7N7 virus NL/219 in Calu-3 cells shown in a previous study, despite differences in epidemiologic profiles and lineages, as well as different known molecular determinants of virulence between these two strains (10). The attenuation of host responses following HPAI H7 virus infection in multiple human cell types, in contrast with the hypercytokinemia often observed with H5N1 viruses, indicates that the suppression of early innate immune responses may represent an alternate mechanism to cause severe disease, as also demonstrated with the reconstructed 1918 virus (9, 10, 21, 29, 46). As many proinflammatory cytokines and chemokines are regulated by
NF-κB, future studies examining the ability of H7 viruses to modulate NF-κB activation, potentially by the activity of the H7 NS1 protein, are warranted (35).

The emergence of HPAI viruses from LPAI precursors typically arises from the insertion or progressive accumulation of multiple basic amino acids at the HA cleavage site (50); to date, only H5 and H7 virus subtypes have demonstrated this capacity. However, viruses detected during the 2012 poultry outbreak in Mexico likely represent an example of an HPAI H7N3 virus emerging as a result of recombination at the HA0 cleavage site. Non-homologous recombination between the HA and NP genes in Chile in 2002, the HA and M genes in British Columbia, Canada in 2004, and the HA and an unidentified protein of eukaryotic origin in Saskatchewan, Canada in 2007 have all demonstrated the ability of H7 influenza viruses to acquire a HPAI phenotype by this mechanism (43, 44, 52). In vitro studies have further identified that H7N3 and H7N7 viruses possessing an insertion of 28S host ribosomal RNA or NP in the HA cleavage site, respectively, is sufficient to confer increased pathogenicity (28, 41). The molecular mechanism of recombination in H7 viruses is not well understood (2, 5, 25), however, the enlarged exposed loop conferred by these insertional events is believed to permit increased accessibility of the HA0 cleavage site (23). Further research is needed to elucidate why the H7 virus subtype appears most susceptible to this form of recombination.

Wild birds represent a reservoir of all influenza A viruses, and surveillance of wild aquatic birds in North America has identified the presence of both H7N3 and H7N9 viruses (22, 31). Frequent reassortment and rapid movement of H7 influenza viruses within this population necessitates continued surveillance of this virus subtype in both wild and domestic birds (18). The continued assessment of H7 viruses detected during routine surveillance, poultry outbreaks, and human infections is critical to both assess the pandemic potential of these strains in humans.
as well as characterize laboratory mammalian models suited to examine the efficacy and cross-reactivity candidate H7 vaccines against viruses within this subtype (15, 24, 26, 42). Because recent H7 subtype viruses associated with poultry outbreaks and surveillance activities possess the ability to cause infection by the ocular route, the use of eye protection to reduce the potential for occupational exposure is prudent (12).

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Figure Legends

Figure 1. Comparison of morbidity and viral replication following influenza A H7 influenza virus infection in mice. BALB/c mice were inoculated i.n. with 10^6 EID50/50μl of each virus indicated. Weight loss (A) was monitored for 14 days following inoculation (5-7 mice/group). MX/7218 weight loss is representative of 6/7 mice which succumbed to infection at this challenge dose. Nose (white bars) and lung (black bars) tissues were collected on days 3 and 6 p.i. following inoculation with HPAI H7N3 viruses (B-C), LPAI H7N3 viruses (D-F), and LPAI H7N9 viruses (G-H). Tissue homogenates (3 mice/group) were titrated in eggs and are expressed as the mean log_{10} EID50/ml plus standard deviation. The limit of virus detection was 10^{1.5} EID50/ml.

Figure 2. Transmissibility of H7N3 and H7N9 influenza viruses among direct contact. Three ferrets were inoculated with 10^6 EID50 of the H7N3 virus MX/7218 or the H7N9 virus gs/NE/11, and nasal washes were collected from each ferret on the indicated days p.i. (left bars) to assess viral replication. A naïve ferret was placed in the same cage as each inoculated ferret 24 h p.i., and nasal washes were collected from each contact ferret on indicated days p.i. (right bars). The limit of virus detection was 10^{1.5} EID50/ml.

Figure 3. Replication kinetics of H7N3 and H7N9 viruses in human respiratory cells. Calu-3 cells were infected at a MOI of 0.01 with the indicated viruses. Supernatants were removed at indicated times p.i., and titers were determined for infectious virus by standard plaque assay. The limit of virus detection was 10 PFU/ml. The mean from duplicate independent cultures per virus plus standard deviation is shown.
Figure 4. Fold regulation of selected genes related to NF-κB signaling in human respiratory cells following HPAI H7N3 virus infection. Calu-3 cells were infected with MX/7218 virus at an MOI of 2. Total RNA was isolated in triplicate from cells at 24 h p.i. and examined by real-time RT-PCR array analysis. Genes which exhibited a significant ($P < 0.05$) >3-fold change in expression over/under that in mock-infected cells are shown. Gray bars signify genes whose regulation is H7 subtype-specific compared with other influenza viruses in this cell type (10).
Table 1. H7 viruses used in this study.

<table>
<thead>
<tr>
<th>Virus origin</th>
<th>Virus Name in this study</th>
<th>Subtype</th>
<th>IVPI(^a)</th>
<th>Virus origin(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/chicken/Chile/180-54/02</td>
<td>ck/Chile/180-54</td>
<td>H7N3</td>
<td>LPAI</td>
<td>Outbreak in broiler breeder flock</td>
</tr>
<tr>
<td>A/chicken/Chile/4322-2/02</td>
<td>ck/Chile/4322-2</td>
<td>H7N3</td>
<td>HPAI</td>
<td>Outbreak in broiler breeder flock</td>
</tr>
<tr>
<td>A/red knot/NJ/1523470/06</td>
<td>rk/NJ/06</td>
<td>H7N3</td>
<td>LPAI</td>
<td>Surveillance of waterfowl and shore birds</td>
</tr>
<tr>
<td>A/chicken/AR/10/08</td>
<td>ck/AR/08</td>
<td>H7N3</td>
<td>LPAI</td>
<td>Surveillance of commercial poultry producer</td>
</tr>
<tr>
<td>A/guinea fowl/NE/17096-1/11</td>
<td>gf/NE/11</td>
<td>H7N9</td>
<td>LPAI</td>
<td>Outbreak in backyard flock</td>
</tr>
<tr>
<td>A/goose/NE/17097-4/11</td>
<td>gs/NE/11</td>
<td>H7N9</td>
<td>LPAI</td>
<td>Outbreak in backyard flock</td>
</tr>
<tr>
<td>A/Mexico/InDRE7218/12</td>
<td>MX/7218</td>
<td>H7N3</td>
<td>HPAI</td>
<td>Human case of bilateral conjunctivitis</td>
</tr>
</tbody>
</table>

\(^{a}\) Pathogenicity phenotype using the Intravenous Pathogenicity Index (IVPI) (40).

\(^{b}\) As described previously (13, 17, 47, 48, 52).
Table 2. Replication of H7 influenza viruses following ocular inoculation in mice.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Day p.i.</th>
<th>rk/NJ/06</th>
<th>gs/NE/11</th>
<th>MX/7218</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye</td>
<td>3</td>
<td>&lt;0.8</td>
<td>2.5 (1/3)</td>
<td>3.4 ± 1.2(2/3)</td>
</tr>
<tr>
<td>Nose</td>
<td>3</td>
<td>&lt;0.8</td>
<td>&lt;0.8</td>
<td>2.9 ± 0.5(2/3)</td>
</tr>
<tr>
<td>Lung</td>
<td>3</td>
<td>&lt;0.8</td>
<td>&lt;0.8</td>
<td>&lt;0.8</td>
</tr>
<tr>
<td>Eye</td>
<td>6</td>
<td>&lt;0.8</td>
<td>2.4 ± 1.0</td>
<td>3.4 ± 0.6</td>
</tr>
<tr>
<td>Nose</td>
<td>6</td>
<td>&lt;0.8</td>
<td>2.2 ± 0.4</td>
<td>3.3 ± 0.7(2/3)</td>
</tr>
<tr>
<td>Lung</td>
<td>6</td>
<td>1.5 (1/3)</td>
<td>&lt;0.8</td>
<td>2.75 (1/3)</td>
</tr>
</tbody>
</table>

Mean virus titers in mice inoculated with $10^6$ EID$_{50}$/5µl of virus following corneal scarification. Virus titers are expressed as the mean log$_{10}$ EID$_{50}$/ml ± standard deviation among mice with positive virus detection (representative of all mice examined unless denoted in parentheses). The limit of virus detection was $10^{0.8}$ EID$_{50}$/ml.
Table 3. Infection of ferrets with H7 influenza viruses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>MX/7218</th>
<th>gs/NE/11</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Observation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Days 1-14 p.i.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight loss(^a)</td>
<td>7.8 (9-10)</td>
<td>nd</td>
</tr>
<tr>
<td>Fever(^b)</td>
<td>2.9 (7)</td>
<td>nd</td>
</tr>
<tr>
<td>Virus in rectal swab(^c)</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Virus titer day 3 p.i.(^d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nasal turbinates</td>
<td>7.2 ± 0.9</td>
<td>6.4 ± 0.1</td>
</tr>
<tr>
<td>Trachea</td>
<td>4.4 ± 2.2 (2/3)</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>Lung</td>
<td>5.0 ± 2.2</td>
<td>3.2 ± 1.2 (2/3)</td>
</tr>
<tr>
<td>Olfactory Bulb</td>
<td>2.8 ± 0.5</td>
<td>2.7 (1/3)</td>
</tr>
<tr>
<td>Brain</td>
<td>2.3 ± 0.2 (2/3)</td>
<td>2.2 (1/3)</td>
</tr>
</tbody>
</table>

**Fold induction of cytokine and chemokine transcript in lungs day 3 p.i.\(^e\)**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>MX/7218</th>
<th>gs/NE/11</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN(\alpha)</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>IL-6</td>
<td>49.4</td>
<td>87.6</td>
</tr>
<tr>
<td>IL-8</td>
<td>173.2</td>
<td>799.7</td>
</tr>
<tr>
<td>IP-10</td>
<td>7.8</td>
<td>6.8</td>
</tr>
</tbody>
</table>

\(^a\)Percent mean maximum weight loss. Parentheses represents the day(s) maximum weight loss was detected p.i. nd, not detected.

\(^b\)Mean maximum rise in body temperature in degrees centigrade (baseline 38.0-38.2°C). Parentheses represents the day(s) maximum rise in temperature was detected p.i.

\(^c\)Number of ferrets with detectable virus in rectal swabs collected on days 3 and 5 p.i.

\(^d\)Virus titers are expressed as the mean log\(_{10}\) EID\(_{50}\)/ml ± standard deviation among ferrets with positive virus detection (representative of all ferrets examined unless denoted in parentheses). The limit of virus detection was 10\(^{1.5}\) EID\(_{50}\)/ml.

\(^e\)Cytokine and chemokine messenger RNA expression in lungs of ferrets (n=3) was measured by RT-PCR and is presented as the mean fold change compared with values in mock-infected animals. Replicate \(C_T\) values for each sample were within 10% of the average.