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Pathogenesis, Transmissibility, and Tropism of a Highly Pathogenic Avian Influenza A(H7N7) Virus Associated With Human Conjunctivitis in Italy, 2013

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Abstract

H7 subtype influenza viruses represent a persistent public health threat because of their continued detection in poultry and ability to cause human infection. An outbreak of highly pathogenic avian influenza H7N7 virus in Italy during 2013 resulted in 3 cases of human conjunctivitis. We determined the pathogenicity and transmissibility of influenza A/Italy/3/2013 virus in mouse and ferret models and examined the replication kinetics of this virus in several human epithelial cell types. The moderate virulence observed in mammalian models and the capacity for transmission in a direct contact model underscore the need for continued study of H7 subtype viruses.

Keywords

mouse; ferret; influenza; transmission; conjunctivitis; cell culture

The emergence of human infection with low-pathogenicity avian influenza (LPAI) A(H7N9) viruses in 2013 and their continued detection in humans to date has highlighted the pandemic potential of the H7 subtype [1]. However, unlike the severe respiratory disease frequently detected among most human cases of A(H7N9) infection, human infection associated with other influenza viruses within the H7 subtype has typically consisted of ocular disease; >80% of confirmed or presumed H7 human infections between 1996 and 2012 have presented with conjunctivitis [2]. The largest such outbreak occurred in 2003 and was caused by high-pathogenicity avian influenza (HPAI) A(H7N7) virus in the Netherlands, with >80 cases of conjunctivitis reported following occupational exposure [3]. More recently, an HPAI A(H7N3) outbreak in Jalisco, Mexico, in 2012 resulted in 2 cases of human conjunctivitis [4]. While most influenza viruses outside the H7 subtype do not

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display an ocular tropism, there remains a need to investigate viruses that cross the species barrier to cause human infection, regardless of the severity of their clinical presentation.

In August and September of 2013, an outbreak of HPAI A(H7N7) virus in the Emilia-Romagna region (Ferrara and Bologna provinces) of Italy resulted in the depopulation and culling of approximately 1.5 million birds [5]. Three polymerase chain reaction–confirmed A(H7N7) infections were reported among individuals who had direct contact with infected poultry, all of whom presented with conjunctivitis; symptoms resolved in the absence of antiviral treatment [5, 6]. Two additional asymptomatic individuals directly involved in culling activities were subsequently identified to be seropositive for A(H7N7) [7]. These cases represent the first evidence of human infection with H7 viruses in Italy since 2002–2003, when 7 individuals exhibited serologic evidence of H7 infection following outbreaks of LPAI H7N3 virus in Brescia and Verona provinces [8]. A subsequent outbreak of HPAI A(H7N7) infection in the Emilia-Romagna region in April and May of 2016, resulting in the death or destruction of >66,000 birds [9], further underscores the importance of studying the capacity of H7 viruses to infect and spread between mammals. Here, we examined in two mammalian models the pathogenicity and transmissibility of an HPAI A(H7N7) virus (A/Italy/3/2013 [hereafter, “Italy/3 virus”]) isolated from a human conjunctival swab specimen and investigated the capacity of Italy/3 virus to replicate in several human cell types.

METHODS

Virus

Italy/3 virus was propagated in the allantoic cavity of 10-day-old embryonated hen’s eggs at 37°C for 26 hours. Allantoic fluid pooled from multiple eggs was clarified by centrifugation and frozen in aliquots at −70°C until use. Determination of infectious titer as 50% egg infectious doses (EID_{50}) or plaque-forming units (PFU) in Madin-Darby canine kidney (MDCK) cells was calculated as previously described [10, 11]. All experiments were conducted under biosafety level 3 containment, including enhancements as required by the US Department of Agriculture and the National Select Agent Program [12].

Mouse Studies

Female BALB/c mice (8 weeks of age) were anesthetized with 2,2,2-tribromoethanol in *tert*-amyl alcohol (Avertin; Sigma-Aldrich) and inoculated intranasally with 10^6 PFU/50 μL of virus [13]. Five mice per group were monitored daily for morbidity (measured by weight loss) and mortality for 14 days after inoculation. Three mice per group were euthanized on days 3 and 6 after inoculation, and lung, nose, and brain tissue specimens were collected and titrated by standard plaque assay in MDCK cells [10]. The 50% mouse infectious dose and 50% mouse lethal dose were determined as previously described [11]. Briefly, mice were inoculated with 10-fold serial dilutions of virus; 5 mice per dilution were observed daily for morbidity and mortality, and 3 mice per dilution were euthanized on day 3 after inoculation, lungs were titered by standard plaque assay, and the infectious dose was determined by the method of Reed and Muench. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention and

were conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited facility.

Ferret Studies

Male Fitch ferrets (Triple F Farms), 7 months of age and seronegative for currently circulating influenza viruses, were housed in cages within a Duo-Flow Bioclean environmental enclosure (Lab Products). Ferrets were inoculated intranasally with 10^6 EID_{50}/mL of virus and monitored daily for clinical signs and symptoms of infection. Transmission of virus to naive ferrets in the presence of direct contact was performed as previously described [14]. Nasal washes (from both inoculated and contact ferrets), conjunctival washes (from inoculated ferrets only), and rectal swabs (from inoculated ferrets only) were collected on alternate days after inoculation and titrated by serial dilution in eggs as previously described [14]. Three additional ferrets were inoculated with virus and euthanized on day 3 after inoculation for the assessment of virus replication and systemic spread, as previously described [13]. Seroconversion of contact ferrets to homologous virus was assessed on day 23 after contact by a standard hemagglutination inhibition assay with 1% turkey red blood cells, as previously described [15].

In Vitro Replication Kinetics

Primary human nasal epithelial cells (PromoCell), alveolar epithelial cells (CellBiologics), or corneal epithelial cells (ATCC) were grown to confluence in transwell inserts (Corning), infected apically in triplicate with virus at a multiplicity of infection (MOI) of 0.01 for 1 hour, washed, and incubated at 37°C in the presence of exogenous TPCK trypsin. The bronchial epithelial cell line Calu-3 (ATCC) was infected similarly to primary cells and incubated at either 37°C or 33°C after infection, without the addition of TPCK trypsin [10]. Aliquots of culture supernatant were removed at indicated times after inoculation, and titers of infectious virus were determined by standard plaque assay in MDCK cells.

RESULTS

Eurasian lineage HPAI H7 viruses typically replicate to high titer in the murine respiratory tract without prior adaptation, causing moderate-to-severe morbidity when mice are inoculated at high doses [13]. Mice inoculated with Italy/3 virus exhibited moderate weight loss but no mortality (Table 1), in agreement with the lack of the mammalian host adaptation marker E627K in the PB2 gene of Italy/3 virus [6]. Viral titers in the nose and lung on days 3 and 6 after inoculation were robust and generally comparable to those of other Eurasian-lineage H7 subtype viruses. The $10^{2.8}$ PFU 50% mouse infectious dose for this virus is generally analogous to those observed for other Eurasian lineage H7N7 viruses, indicating that this virus may possess similar infectivity as compared to other viruses within this subtype [13].

Owing to the close physiologic similarity of human and ferret respiratory tracts and similar presentation of signs and symptoms of infection, ferrets represent the best small-mammalian model to study the pathogenicity and transmissibility of influenza viruses [16]. Ferrets inoculated with Italy/3 virus exhibited a generally mild infection, with low and transient

weight loss and fever detected early after infection (Table 1). Despite the limited morbidity observed in infected ferrets, virus replicated to high titers throughout the upper and lower respiratory tract, including the lung. Low viral titers were found in rectal swabs of all inoculated ferrets and in intestinal tissue of 1 of 3 ferrets. Virus was further detected in the olfactory bulb and anterior brain of 2/3 ferrets. Italy/3 virus was present in conjunctival washes from 1 of 3 ferrets observed throughout the acute phase of infection but was not detected in the eye or surrounding conjunctiva from ferrets scheduled for postmortem necropsy on day 3 after inoculation (Table 1; data not shown), similar to what was observed following ferret inoculation with HPAI H7N3 virus associated with ocular disease in humans [14]. Overall, the distribution and magnitude of viral titers were generally in accord with those of other low-to-moderately virulent H7 viruses in the ferret model [13, 14, 17].

H7 viruses of both Eurasian and North American lineages associated with human conjunctivitis have been shown to demonstrate limited virus transmissibility in the presence of direct contact. To determine whether Italy/3 virus shared this property, we placed naive ferrets in direct contact with virus-inoculated ferrets 24 hours after inoculation and collected nasal washes from both inoculated and contact ferrets on alternate days to assess virus transmissibility (Figure 1A). Ferrets inoculated with Italy/3 virus shed detectable virus in nasal washes at titers of $>10^4$ EID_{50} through day 5 after inoculation, typical of avian influenza viruses in this model. One of 3 ferrets placed in direct contact shed virus with comparable kinetics and titer as inoculated ferrets and seroconverted to homologous virus; a second ferret seroconverted but did not shed detectable virus (Figure 1A and data not shown). These results indicate that, similar to other HPAI H7 viruses associated with human conjunctivitis, Italy/3 virus possesses a capacity to transmit virus in a direct contact ferret model, albeit inefficiently.

The detection of infectious virus in both upper and lower respiratory tract tissues of ferrets prompted us to determine the capacity of Italy/3 virus to replicate in epithelial cells distributed throughout the human respiratory tract, as well as the eye. While the receptor binding profile of Italy/3 virus has not been determined, previous Eurasian-lineage A(H7N7) viruses associated with human infection have maintained strong binding to α2–3 linked sialic acids [15]. To examine differences in tissue tropism, we infected 3 primary human cell types with Italy/3 virus: nasal, alveolar, and corneal epithelial cells (Figure 1B). Italy/3 virus exhibited productive replication in all cell types; peak titers of $>10^4$ PFU/mL were detected 48–72 hours after inoculation in nasal epithelial and corneal epithelial cells, and peak titers of $>10^3$ PFU/mL were detected in alveolar epithelial cells 48 hours after inoculation. Robust replication was further observed in the human bronchial epithelial cell line Calu-3 (Figure 1C), with viral titers reaching $>10^7$ PFU/mL by 48 hours after inoculation when cultured at 37°C and virus exhibiting a temperature sensitivity at 33°C that is common among avian influenza viruses [17, 18]. These results demonstrate that, similar to other avian influenza viruses, Italy/3 virus is capable of replicating in upper respiratory tract, lower respiratory tract, and ocular epithelial cell types that possess differing distributions of terminal sialic acids on their cell surface.

DISCUSSION

HPAI H7 influenza viruses of both Eurasian and North American lineages continue to cause infrequent outbreaks in poultry, resulting in extensive depopulations of infected and at-risk birds, profound economic losses, and potential occupational exposures of individuals involved with outbreak control. Over 80 distinct outbreaks of HPAI and/or LPAI H7 virus in poultry have been reported to the World Organization for Animal Health in the past decade worldwide, highlighting the pandemic potential of this virus subtype and the potential for human infection to occur as a result of occupational exposure during culling and depopulation activities. While H7 viruses have not demonstrated sustained human-to-human transmissibility, clusters of limited transmission among close contacts with both HPAI A(H7N7) and LPAI A(H7N9) viruses has emphasized the need to monitor the ability of H7 viruses to acquire this property [1, 3]. Furthermore, while all in vivo experiments were conducted following traditional intranasal virus inoculation, it is likely that, similar to other human and avian influenza viruses tested in the ferret model, Italy/3 virus would maintain infectivity following ocular exposure [19, 20]. The mammalian pathogenicity and transmissibility of the HPAI A(H7N7) Italy/3 virus appears to be generally comparable to observations for other HPAI H7 viruses from both lineages associated with human conjunctivitis, underscoring the need to monitor influenza viruses for which conjunctivitis is the primary manifestation of disease.

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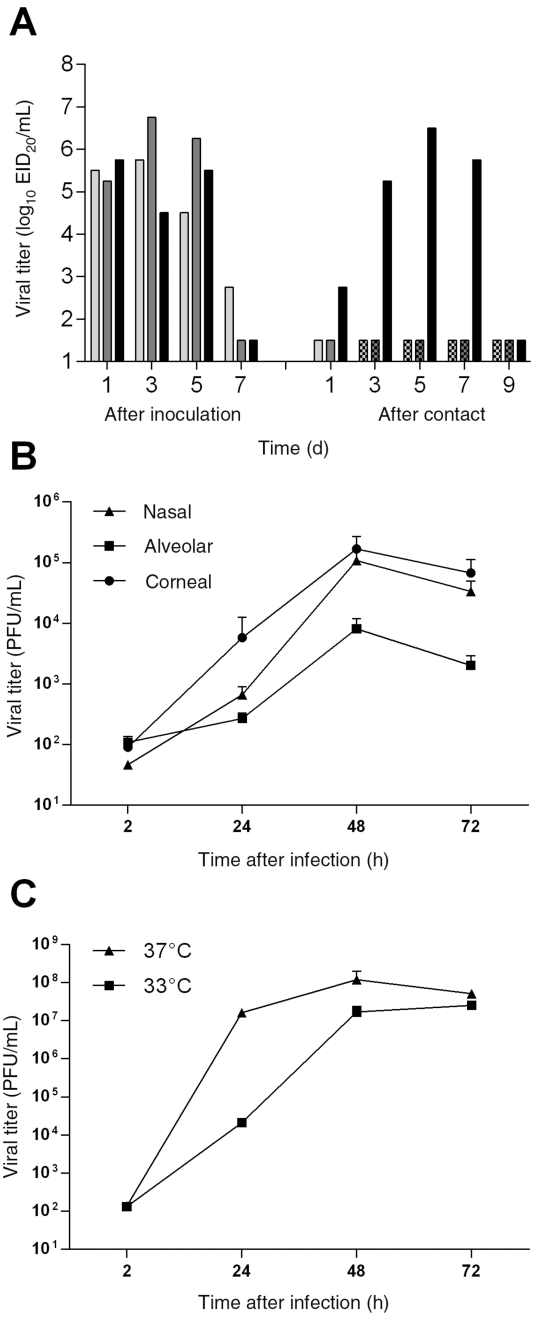

Figure 1.

Replication of influenza Italy/3 virus in vivo and in vitro. *A*, Three ferrets were inoculated with 10^6 50% egg infectious doses (EID_{50}) of Italy/3 virus, and nasal washes were collected from each ferret on the indicated days after inoculation (left bars) to assess viral replication. A naive ferret was placed in the same cage as each inoculated ferret at 24 hours after inoculation, and nasal washes were collected from each contact ferret on the indicated days after contact (right bars). The limit of virus detection was $10^{1.5}$ EID_{50}/mL. Seroconversion to homologous virus was detected in the first and third ferret contact pairs shown on day 23 after contact. *B*, Primary human nasal epithelial, alveolar epithelial, or corneal epithelial

cells were infected apically with Italy/3 virus at a multiplicity of infection of 0.01 for 1 hour, washed, and incubated at 37°C. Viral titers were determined at the indicated times. The limit of virus detection was 10 plaque-forming units (PFU)/ mL. The mean value (+SD) from triplicate independent cultures per virus is shown. *C*, Calu-3 cells were infected similarly to primary cells and incubated at 37°C or 33°C. Supernatants were removed at indicated times after inoculation, and titers of infectious virus were determined by standard plaque assay at the indicated times. The limit of virus detection was 10 PFU/mL. The mean value (+SD) from triplicate independent cultures per virus is shown.

Table 1

Characteristics of Influenza A/Italy/3/13 Virus Infection in Mice and Ferrets

Variable	Value
Mice	
MID_{50}, PFU/50 μL	$10^{2.8}$
MLD_{50}, PFU/50 μL	$>10^{6}$
Maximum percentage weight loss, mean[a]	14.2
Virus titer, $\log_{10}$ PFU/mL, by time after infection[b]	
3 d	
Nose	3.0 ± 0.6
Lung	5.6 ± 0.2
6 d	
Nose	3.4 ± 1.9
Lung	6.3 ± 0.1
Ferrets[c]	
Maximum percentage weight loss, mean	4.6
Maximum increase in body temperature, °C, mean[d]	1.2
Virus detected in CW, proportion	1/3
Virus detected in RS, proportion	3/3
Virus titer 3 d after infection, $\log_{10}$ EID_{50}/mL[e]	
Nasal turbinates	7.5 ± 0.3
Trachea	5.3 ± 0.6
Lung	4.1 ± 1.8
Olfactory bulb	3.1 ± 0.2
Brain	3.3 ± 1.0
Intestine	2.8

Data are mean ± SD, unless otherwise indicated.

Abbreviations: CW, conjunctival washes; MID_{50}, 50% mouse infectious dose; MLD_{50}, 50% mouse lethal dose; PFU, plaque-forming units; RS, rectal swabs.

[a] Data are from d 7 after infection.

[b] Data are from 3 animals per group. The limit of virus detection was 10 PFU.

[c] Data are from 2 d after infection, for weight loss; 1–2 d after infection, for fever; 1 and 3 d after infection, for virus detection in CW; 3 and 5 d after infection, for virus detection in RS; and 3 d after infection, for virus titers.

[d] Baseline body temperature range, 38.6°C–38.9°C.

[e] Data for nasal turbinates, trachea, and lung are from 3 of 3 ferrets, data for olfactory bulb and brain are from 2 of 3 ferrets, and data for intestine are from 1 of 3 ferrets. The limit of virus detection was $10^{1.5}$ EID_{50}/mL.