Sciencexpress Reports

Infectivity, Transmission, and Pathology of Human H7N9 Influenza in Ferrets and Pigs

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The emergence of the H7N9 influenza virus in humans in Eastern China has raised concerns that a new influenza pandemic could occur. Here, we used a ferret model to evaluate the infectivity and transmissibility of A/Shanghai/2/2013 (SH2), a human H7N9 virus isolate. This virus replicated in the upper and lower respiratory tracts of the ferrets and was shed at high titers for 6 to 7 days, with ferrets showing relatively mild clinical signs. SH2 was efficiently transmitted via direct contact, but less efficiently by airborne exposure. Pigs could be productively infected by SH2 and shed virus for 6 days but were unable to transmit the virus to other animals. Under appropriate conditions human-to-human transmission of the H7N9 virus may be possible.

On 31 March 2013, the Chinese National Health and Family Planning Commission announced the occurrence of three human infections with H7N9 subtype influenza viruses (*1*). Analyses of the sequences of the human H7N9 isolates indicates that the virus was derived by reassortment events between H7 and N9 subtype viruses, possibly from aquatic birds, and enzootic H9N2 viruses from chickens (*1*, *2*). As of 1 May 2013, over 125 human cases have been confirmed, with the majority of the patients hospitalized and many suffering acute respiratory distress syndrome (ARDS) (*3*–*5*). Over 75% of human cases had a history of contact with, or exposure to poultry before disease onset (*4*), suggesting a zoonotic origin of the infections. Identification of some family clusters raised concerns of human-to-human transmission by the H7N9 virus (*4*). Sequence analyses showed that the H7N9 viruses might have undergone mutations that are favorable for efficient replication in mammalian hosts (*2*, *6*–*8*).

To characterize this H7N9 virus, its infectivity, transmissibility, and pathogenicity were assessed in ferrets, the primary mammalian model for human influenza. Influenza free ferrets (n = 6) were inoculated intranasally with 10^6 50% tissue culture infectious dose ($TCID_{50}$) of A/Shanghai/02/2013 (SH2), a human isolate from a fatal index case (*1*). These ferrets displayed a brief fever at one to two days post-inoculation (dpi) and robust sneezing and nasal discharge throughout the experiment (Table 1 and fig. S1, A, C, and D). Coughing and mild lethargy occurred periodically during the course of the disease, but weight change was essentially negligible (fig. S1, B, E, and F). Ferrets (n = 6) inoculated with the pandemic A(H1N1) 2009 virus, A/California/07/2009 (CA07), showed similar signs as the SH2 inoculated animals, except for more prominent nasal discharge and slightly greater weight loss but without statistically significant differences (fig. S1). All animals in both virus groups displayed near normal activity levels by 14 dpi but had some residual sneezing and nasal discharge. SH2 infected animals were normal for body temperature, body weight, sneezing, coughing and activity by 16 dpi (table S1).

The virus load in the nasal washes of each inoculated ferret was determined daily by $TCID_{50}$ assays in Madin-Darby Canine (MDCK) cells. Virus shedding was detected at one dpi and sustained at high titers (3.1-5.4 log $TCID_{50}$/ml) for 7 days (Fig. 1A and Table 1). Thus, virus shedding occurred before the development of most clinical signs. For the CA07 infected ferrets, virus shedding began at one dpi and continued at high titers (3.1-5.9 log $TCID_{50}$/ml) for 6 days (Fig. 1A). Overall, SH2 and CA07 infection showed comparable clinical profiles and virus shedding kinetics in ferrets with no statistically significant differences.

Efficient transmission of influenza viruses in ferrets is considered as a predictor of human-to-human transmissibility (*9*). Six ferrets inoculated with 10^6 $TCID_{50}$ of virus were placed, two each, in three transmission cages. At one dpi, a naïve ferret was introduced into each cage with the inoculated ferrets to measure direct contact transmission (fig. S2A). An additional naïve ferret was placed in an adjacent cage, separated by a distance of 10 cm for airborne exposure (fig. S2A), with airflow toward this cage at a rate of <0.2 m/second. An identical experiment was conducted with the CA07 virus.

All three direct contact ferrets of the SH2 inoculated group shed virus within 3 days post-exposure (dpe) and showed sneezing, nasal discharge, cough and inactivity by 6 dpe, but only one developed fever for one day at 4 dpe (Fig. 1B and Table 1). One of the airborne exposed ferrets began shedding virus at 3 dpe and continued shedding virus at high titers for 6 days (Fig. 1C and Table 1). The two remaining airborne exposed ferrets did not shed detectable virus and had few clinical signs (Fig. 1C and Table 1). All inoculated and direct contact ferrets seroconverted by 14 dpi or 14 dpe. The airborne exposed ferret that shed virus seroconverted with an HAI titer of 1:320, while one airborne exposed ferret that did not shed virus had an HAI titer of 1:40 at 14 dpe (Table 1). Thus, ferrets infected with SH2 can transmit the virus via direct contact and airborne exposure, albeit the latter less efficiently. All naïve ferrets placed in CA07 direct contact and airborne exposure cages began shedding virus between one and four dpe (Fig. 1, D and E, and Table 1), consistent with prior studies (*10*, *11*).

Eighteen ferrets, inoculated with SH2 as above, were sacrificed at 1, 3, 5, 7, 10, and 14 dpi to examine the gross pathology and infected tissue types to study disease progression. Respiratory and other major organs were collected for histopathologic examination and immunostaining for

the presence of viral nucleoprotein (NP). Virus load was also determined by detection of viral Matrix (M) gene by real-time PCR and $TCID_{50}$ assays.

Gross pathological examination of the lungs revealed multi focal lesions on days 3, 5 and 7, with day 7 showing the most severe pathology. At 3 dpi, histopathological examinations showed acute inflammatory infiltrates in the alveoli and bronchioles, with predominant infiltration by neutrophil cells (Fig. 2, A and D). By 5 and 7 dpi more prominent lymphocytic and plasmocytic cells infiltration could be seen within the alveoli and bronchioles (Fig. 2, B, C, and E).

NP-positive cells were detected in the respiratory epithelial cells of the nasal turbinate and trachea at 3 dpi (Fig. 2, F and G). Bronchiolar epithelial cells were also positive for NP on 3 dpi (Fig. 2H). Viral RNA was detected in ferret nasal turbinate, trachea, lungs, hilar lymph nodes and brain (Fig. 3) and the presence of infectious virus was confirmed by $TCID_{50}$ assays (fig. S3) and NP staining in hilar lymph nodes and brain (fig. S4). Thus, inoculation of SH2 can result in infection of the upper and lower respiratory tracts, and lymph nodes and brain. A different site of inoculation, such as intratracheal (*12*), may result in a different pathology. Clinically, the cellular tropism of H7N9 viruses may determine its spectrum of clinical disease and it may be advisable to examine human cases for signs of central nervous system affects.

The domestic pig is a major mammalian host of influenza A viruses, and may have played a key role in facilitating the emergence of human pandemic influenza viruses (*13*). To evaluate the infectivity and transmissibility of SH2 in pigs, four animals were inoculated with 10^6 $TCID_{50}$ of SH2. Virus shedding was detected as early as 1 dpi and lasted for 5 to 6 days; with peak virus titers ranging from 3.49 to 5.16 log $TCID_{50}$/ml (Table 2 and fig. S5A). Sneezing, nasal discharge and diminished activity were observed from one to 1.5 days following virus shedding.

To determine transmissibility, at 1 dpi, two naïve pigs were housed with each of the two groups of inoculated animals, and a further naïve pig and ferret were placed in separate cages to assess airborne transmission (fig. S2B). None of the direct contact pigs or airborne exposed pigs and ferrets shed virus via the nasal or rectal routes (Table 2 and fig. S5, C and D). One of the four direct contact pigs and both airborne exposed ferrets seroconverted by 14 dpe. Neither airborne exposed pig seroconverted (Table 2). Three additional inoculated pigs were sacrificed at 4 dpi to detect the presence of virus in major organs. Viral RNA was detected in the nasal turbinate, trachea, lungs and lymph nodes of these pigs and NP in nasal turbinates (fig. S6). An identical transmission experiment using the CA07 virus showed that all direct contact pigs and airborne exposed pigs and ferrets could shed virus (Table 2 and fig. S5, B to D). Thus, SH2 could productively infect domestic pigs after intranasal inoculation.

Here we demonstrated that ferrets can be infected by SH2 and shed virus that could transmit to direct contact and airborne exposed animals, resulting in productive infections. Shedding of this virus occurred before the development of the majority of clinical signs. This trait has been observed previously for pandemic and seasonal influenza (*10*, *11*, *14*, *15*). If this virus acquires the ability to efficiently transmit from human-to-human, extensive spread of this virus may be inevitable, as quarantine measures will lag behind its spread. Continued prevalence of the H7N9 virus could lead to it becoming endemic in poultry as has occurred with the Asian highly pathogenic H5N1 and H9N2 virus lineages (*16*, *17*). If so, the opportunities for the H7N9 virus to evolve to acquire human-to-human transmissibility, or to be introduced to pigs, would greatly increase. To prevent this from happening, it may be advisable to reconsider the management of live poultry markets, especially in the urban areas.

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Acknowledgments: We gratefully acknowledge our colleagues from the Joint Influenza Research Center (SUMC/HKU) and the State Key Laboratory of Emerging Infectious Diseases for their excellent technical assistance and D. K. Smith for editorial assistance. This study was supported by the National Institutes of Health (NIH, National Institute of Allergy and Infectious Diseases contract HSN266200700005C), Li Ka Shing Foundation, the Area of Excellence Scheme of the University Grants Committee of the Hong Kong SAR (grant AoE/M-12/06), Shenzhen Peacock Plan High-End Talents

Program (KQTD201203), and Emergency Research Project on human infection with avian influenza H7N9 virus from the National Ministry of Science and Technology (no. KJYJ-2013-01-01-01 to Dr. Shu).

Supplementary Materials
www.sciencemag.org/cgi/content/full/science.1239844/DC1
Materials and Methods
Figs. S1 to S6
Table S1
References (*18–21*)

30 April 2013; accepted 20 May 2013
Published online 23 May 2013
10.1126/science.1239844

Table 1. Transmissibility of SH2 and CA07 viruses in ferrets.

Ferret groups	Virus*	Signs	Shedding of virus			Seroconversion‡ #animals (titers)
			# Animals	Onset†	Duration (days)	
Inoculated	SH2	6/6	6/6	1,1,1,1,1,1	7,7,7,7,7,7	6/6 (320-640)
	CA07	6/6	6/6	1,1,1,1,1,1	5,6,6,6,6,6	6/6 (>1280)
Direct contacts	SH2	3/3	3/3	2,3,3	5,6,7	3/3 (320-640)
	CA07	3/3	3/3	1,1,2	6,6,6	3/3 (>1280)
Airborne exposed	SH2	3/3	1/3	–,–,3	–,–,6	2/3 (320, 40, <)
	CA07	3/3	3/3	2,3,4	6,6,7	3/3 (>1280)

*SH2: A/Shanghai/2/2013(H7N9), CA07: A/California/07/2009 (H1N1).
†dpi, dpe.
‡HAI titer of post-exposure sera at 14 dpi or 14 dpe for each animal, starting dilution at 1:10. "–," undetectable; "<," <1:10.

Table 2. Transmission of SH2 and CA07 in pigs and to ferrets.

Infection group	Virus*	Shedding of virus			Seroconversion‡ #animals (titers)
		#Animals	Onset†	Duration (days)	
Inoculated pigs	SH2	4/4	1,2,2,2	5,5,5,6	3/4 (10,40,80,640)
	CA07	4/4	1,1,1,1	6,6,6,7	3/4 (20,40,80,80)
Direct contact pigs	SH2	0/4	–	–	1/4 (<,<,<,320)
	CA07	4/4	3,3,3,4	5,6,6,7	4/4 (40,40,80,80)
			Airborne exposed		
Pigs	SH2	0/2	–	–	0/2 (<,<)
	CA07	2/2	3,4	5,6	2/2 (40,160)
Ferrets	SH2	0/2	–	–	2/2 (40,320)
	CA07	2/2	6,7	7,7	2/2 (>1280, >1280)

*SH2: A/Shanghai/2/2013(H7N9), CA07: A/California/07/2009 (H1N1).
†dpi, dpe.
‡HAI titer of post-exposure sera at 14 dpi or 14 dpe for each animal, starting dilution at 1:10. "–," undetectable; "<," <1:10.

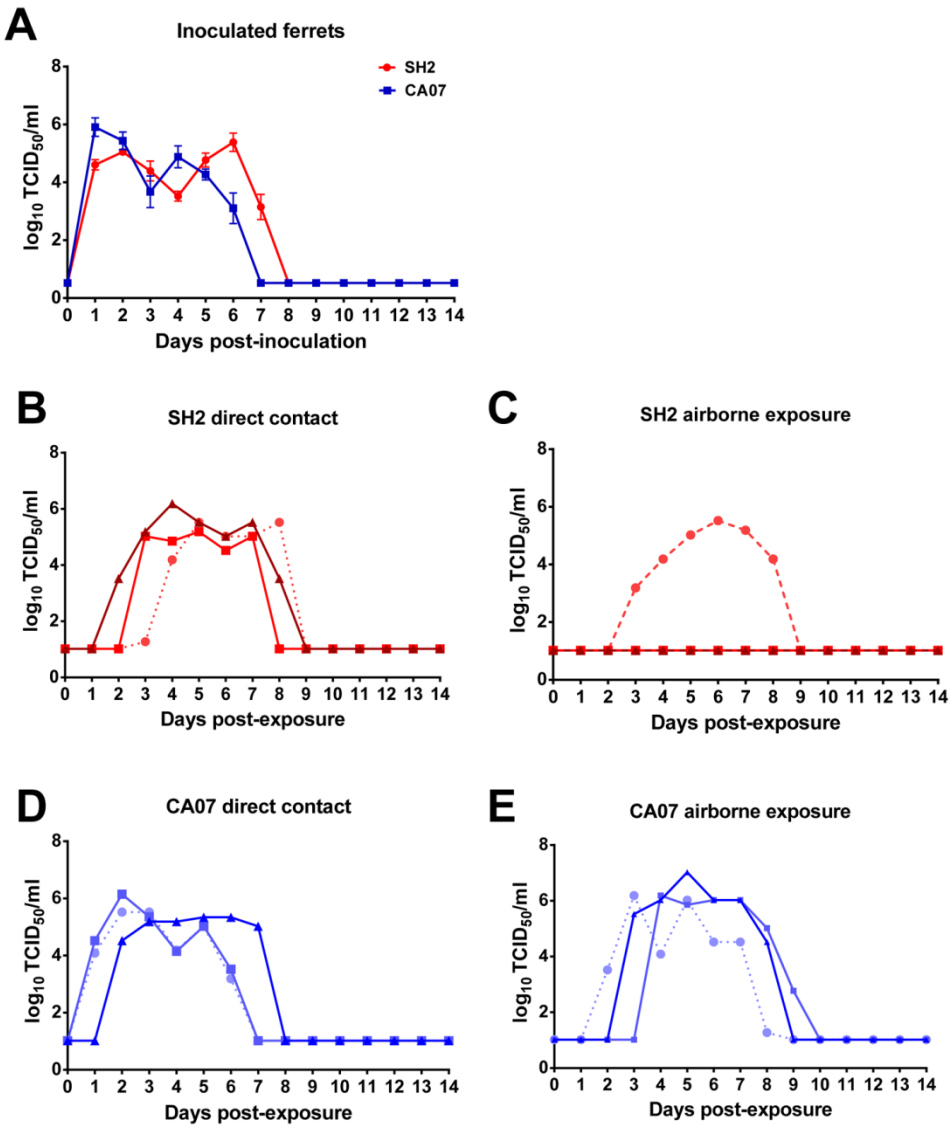

Fig. 1. Shedding of virus by inoculated and exposed ferrets. Nasal washes from ferrets inoculated with or exposed to SH2 or CA07 were tested for infectious virus titer over a 14-day period following intranasal inoculation (**A**), direct contact (**B** and **D**), or airborne (**C** and **E**) exposure. Results are expressed as $\log_{10}$ $TCID_{50}$/ml. Panel (A) shows virus titers for six ferrets (mean ± SEM) while panels (B) to (E) show virus titers for individual ferrets.

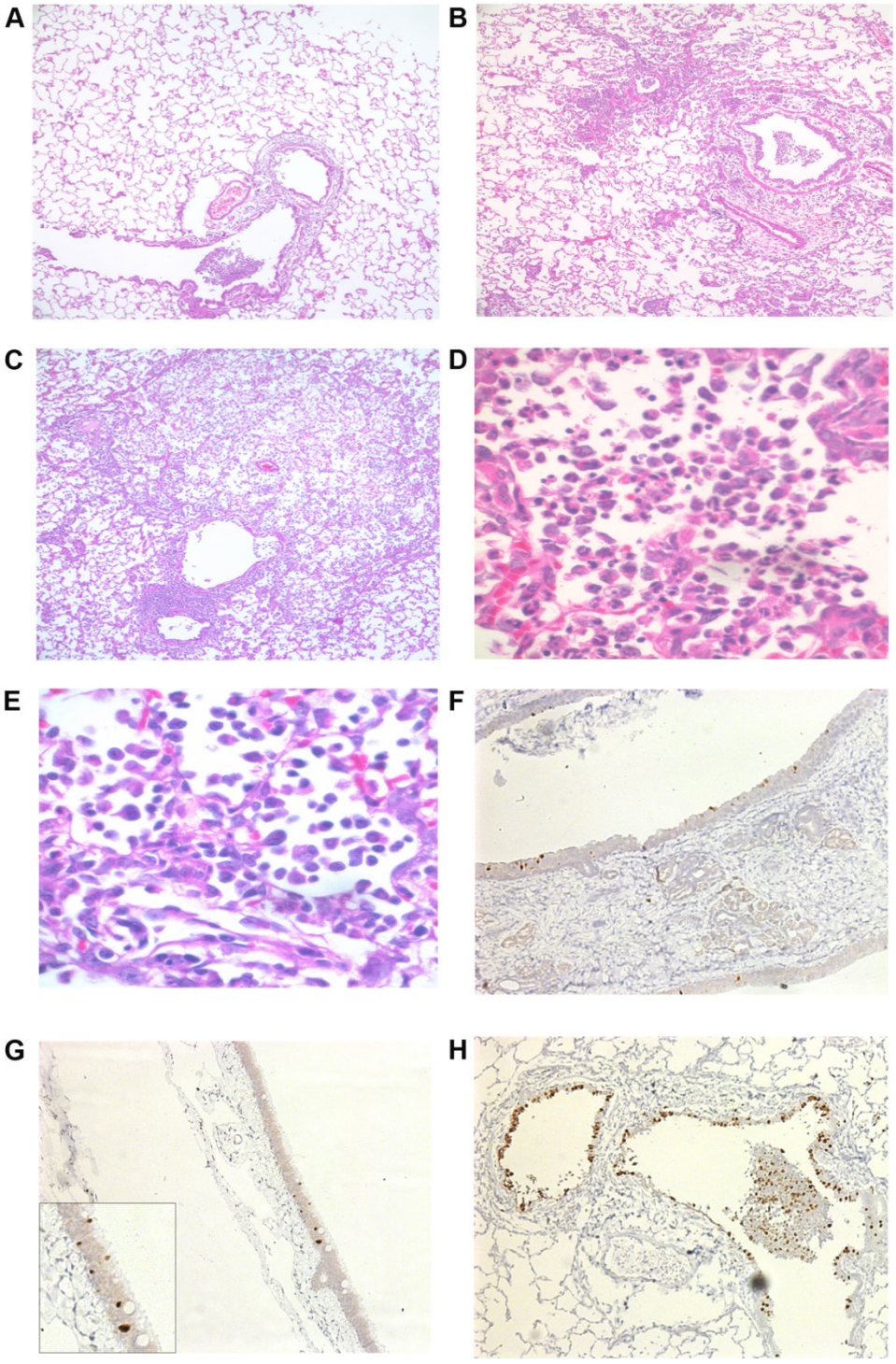

Fig. 2. Histopathology and immunohistochemical analyses of ferret respiratory tissues following SH2 infection. Pulmonary tissues were harvested from ferrets infected with SH2 and hematoxylin and eosin stained at 3 days post-infection (dpi) (**A**), 5 dpi (**B**), and 7 dpi (**C**). Pathological changes, especially bronchopneumonia, with a mix of acute inflammatory infiltrates in the alveoli and bronchioles were observed. At 3 dpi, neutrophils were the predominant infiltrating cell type (**D**). By 5 dpi, chronic lymphocytic and plasmocytic infiltrates are more prominent (**E**). Influenza nucleoprotein (NP) antigen staining is visible as brown color in the nasal turbinate (**F**), trachea (**G**), and lung (**H**) at 3 dpi.

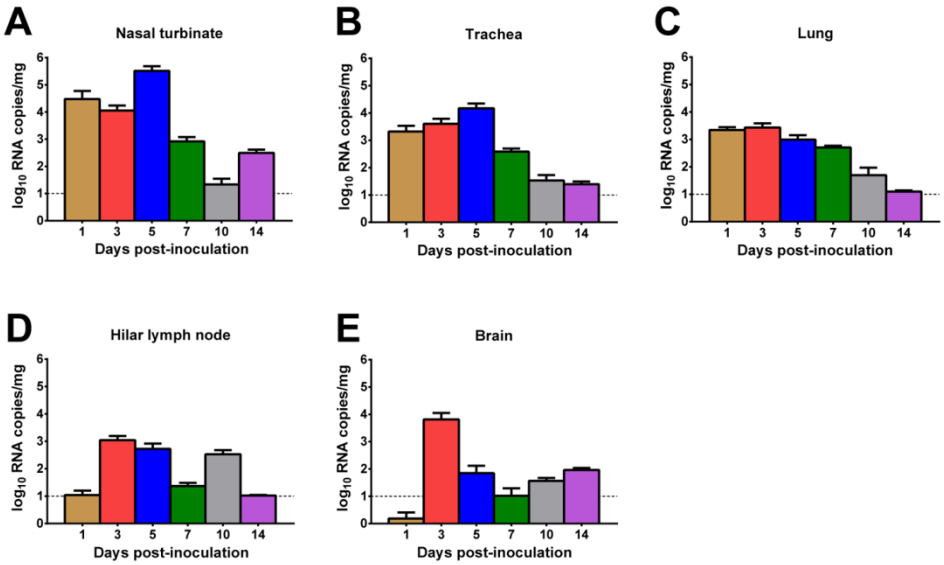

Fig. 3. Detection of influenza virus RNA in ferret tissues following intranasal inoculation with SH2. Nasal turbinate (**A**), trachea (**B**), lungs (**C**), hilar lymph node (**D**), and brain (**E**) and other major organs were harvested (n = 3) at various time points post inoculation. Tissue homogenates were processed for RNA extraction and quantitative RT-PCR to detect the influenza virus Matrix gene. Results are mean ± SEM. The dotted line represents the limit of detection. Samples collected at 1, 3, 5, 7, 10, and 14 dpi from serum, spleen, intestine, mesenteric lymph node, liver, heart, and kidney were all under the limit of detection.