Human H7N9 Influenza A Viruses Replicate in Swine Respiratory Tissue Explants

Jones J.C.1*, Baranovich T1*, Zaraket H1, Guan Y2,3,4, Shu Y5, Webby RJ1, Webster RG1#

1 Department of Infectious Diseases, St. Jude Children’s Research Hospital, Memphis, TN, USA; 2 Joint Influenza Research Centre [Shantou University Medical College (SUMC)/University of Hong Kong (HKU)], Shantou University, Shantou, PR China; 3 State Key Laboratory of Emerging Infectious Diseases (HKU-Shenzhen Branch), Shenzhen Third People’s Hospital, Shenzhen, PR China; 4 State Key Laboratory of Emerging Infectious Diseases/Centre of Influenza Research, School of Public Health, The University of Hong Kong, Hong Kong SAR, PR China; 5 National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Key Laboratory for Medical Virology, National Health and Family Planning Commission, Beijing, PR China.

* These authors contributed equally to the study

Key words: human H7N9 influenza virus, swine, respiratory explants

Word count: abstract (75), main text (1375)

*Corresponding author:
Robert G. Webster
Department of Infectious Diseases
St. Jude Children’s Research Hospital
262 Danny Thomas Place, Memphis, TN 38105-3678.
Phone: (901) 595-3400
Fax: (901) 595-8559
E-mail: robert.webster@stjude.org
Abstract

Recently, novel H7N9 influenza viruses have caused an unprecedented outbreak in humans. Pigs are an important intermediate host for influenza; thus we assessed the replication ability of three human H7N9 viruses (A/Anhui/1/2013, A/Shanghai/1/2013, A/Shanghai/2/2013) in swine tissue explants. All viruses tested replicated efficiently in tracheas and bronchi of the explants, with limited replication in alveolar cells. Swine respiratory tissue explants can serve as an efficient model for screening replication potential of newly emerging H7N9 viruses.
In the spring of 2013, human infections with novel avian-origin H7N9 influenza viruses were reported in China (1) marking the first time this subtype has appeared in humans. One hundred and thirty-four human infections have been confirmed with a resulting mortality rate of approximately 32% (2-4). Identification of the source of the viruses is currently under investigation, but exposure to infected domestic poultry has been indicated in the majority of the cases. At present, human-to-human transmission is unsustained (5).

The H7N9 viruses contain genes solely of avian origin, with surface proteins donated from ducks or other aquatic waterfowl, and internal proteins from chickens or other migratory wild birds (2, 6). Currently, there are two lineages of H7N9 viruses reported; with A/Anhui/1/13-like viruses possessing molecular markers associated with adaptation to humans: mutations in the receptor binding hemagglutinin (HA) protein confer enhanced binding to mammalian receptors (S138A, G186V, and Q226L (H3 numbering), and the E627K mutation in the PB2 that facilitates replication of avian influenza viruses in mammals (2, 7). A/Shanghail/1/13-like viruses lack these markers. But all H7N9 viruses have high nucleotide substitution rates and positive selective pressure at the protein level (8), in addition to the inherent high reassortment capacity of influenza viruses. Collectively, these characteristics could lead to generation of a pandemic virus capable of spreading not only from birds to humans but also from humans to other humans.

Swine have long been recognized as an intermediate host for influenza viruses and can facilitate the adaptation and movement of avian viruses into humans (9). Most recently, swine played a critical role in the genesis of the 2009 pandemic H1N1 virus (10). To date, no H7N9 infections from the recent outbreak have been reported in swine. Surveillance of swine farms in the affected regions have yielded over 4000 animal or environmental samples, all of which have
been negative (5). These viruses have already shown the ability to infect humans, and the potential that they may infect swine and generate additional mammalian adaptations is a significant public health concern. Recent studies by Zhu et al. demonstrate that a single human H7N9 isolate was able to infect pigs experimentally, but was unable to transmit to cage mates (11). For further risk assessment and understanding of the pathogenesis of H7N9 viruses in swine, evaluation of additional human H7N9 isolates is warranted. However, studies that involve a large animal model, such as swine, can be cumbersome and expensive. Recently, models have been developed for culture and infection of swine respiratory tissues ex vivo. Importantly, these respiratory tissue explants retain their structure and receptor specificity and mimic infection patterns observed in live animals (12-14). Furthermore, these ex vivo studies provide for a rapid and humane option to test larger numbers of influenza virus isolates to pre-select virus candidates for or as a substitute to live animal studies.

Here, we have examined the ability of three genetically distinct human H7N9 isolates, A/Anhui/1/2013 (Anhui/1), A/Shanghai/1/2013 (Shang/1) and A/Shanghai/2/2013 (Shang/2) to replicate in swine upper airway (trachea) or lower airway (lung) tissue explants. Viruses Anhui/1, Shang/1, and Shang/2 were isolated from patients who progressed to severe respiratory distress and ultimately death (15). As controls, we used A/Swine/Missouri/2124514/06 (Swine/MO, H2N3), a swine virus isolate for which replication in pigs has since been recapitulated experimentally (16), and A/Duck/NewJersey/7872-27/95 (Duck/NJ, (H2N2), an avian virus that does not replicate in swine tissues (Jones JC, unpublished data). Viruses were propagated and titrated in embryonated chicken eggs and a 50% egg infectious dose (EID<sub>50</sub>) was determined. Tissue samples were obtained from 1-wk-old piglets, and respiratory tissue explants (5mm) were prepared as previously described (12, 14). The explants were maintained on
transwell inserts (Corning, Tewksbury, MA) with the basal chamber containing BEBM medium supplemented with SingleQuot growth factors (Lonza, Walkersville, MD). After explant preparation, the medium was replaced every three hours for a total three changes in order to remove cytokines and they were incubated at 37°C in 5% CO₂ for >18 h before inoculation. The explants were washed extensively with PBS prior to inoculation with 10⁶ EID₅₀ units/explant. After a 1 h adsorption, the explants were washed 2x with 0.9% NaCl, pH 2.2 and 3x with PBS. For each time point indicated, 300 µl of infection medium (BEBM + 0.5% bovine serum albumin) was added to the apical surface for 30 min, harvested, and titrated on MDCKs, with 50% tissue culture infectious dose (TCID₅₀) determined using the method of Reed and Muench (17). Tissues were sampled every 24 h for 3 days. Data are representative of 3 or 4 independent experiments with two or more explants per tissue and per virus.

As expected, Swine/MO was able to productively replicate in both tracheal and lung explants [FIG 1 A, B]. The negative control avian virus, Duck/NJ, failed to replicate in either tissue at any time point sampled. The three human H7N9 viruses tested replicated efficiently in both tracheal and lung explants with the highest virus titers detected at 48 or 72 h post-inoculation (hpi). Notably, there was a statistically significant delay in replication of Shang/1 virus at 1 hpi (p<0.01) and Anhui/1 at 24 hpi (p<0.001) in tracheal explants when compared to the control H2N3 swine virus [FIG 1A]. All viruses replicated to statistically indistinguishable levels in the lung explants apart from Duck/NJ [FIG1B]. Importantly, all detectable virus titers at 48 and 72 hpi were significantly higher to those of carryover titers detected at 1 hpi (p≤0.01).

All human H7N9 viruses and Swine/MO infected tissues displayed positive influenza nucleoprotein (NP) antigen staining as expected. NP staining was detected in tracheal epithelia inoculated with influenza Anhui/1, Shang/1, Shang/2, and Swine/MO [FIG 2]. In lung explants,
viral antigen staining was largely restricted to the bronchi, with the exception of Anhui/1, Shang/2 and Swine/MO, where limited positive viral antigen staining was detected in cells of the alveoli adjacent to infected bronchi. In contrast, no viral antigen staining was detected in either tracheal or lung explants inoculated with avian influenza Duck/NJ virus. Several factors contribute to the important role that swine play in the genesis and dissemination of novel influenza viruses: 1) both human-like α2,6- and avian-like α2,3-linked sialosides are present in the porcine respiratory tract; 2) experimental studies have confirmed the susceptibility of pigs to both avian and human influenza viruses (12) and 3) pigs commonly interact with both humans and avian species in agricultural settings. The emergence of avian H7N9 viruses that are capable of infecting humans and that possess multiple molecular markers associated with adaptation to mammals is unprecedented and troubling. To date, there is little evidence of human-to-human transmission of H7N9; however, the potential circulation of this virus in pigs may provide critical changes that alter pathogenicity or transmission potential. In this study, we build upon in vivo observations from Zhu et al. who demonstrated a single H7N9 isolate (A/Anhui/1/13) is able to replicate in pigs to peak titers between 3.5 and 5.6 $\log_{10}$ TCID$_{50}$/ml (11). We have demonstrated ex vivo that 3 human H7N9 viruses, including the genetically disparate Shang/1, is able to replicate in both the upper and lower respiratory tract of pigs with peak titers ranging 6.0-7.2 $\log_{10}$ TCID$_{50}$/ml. The slightly higher titers seen with the explants when compared to in vivo data (11) may be due to differences in relative inocula and tissue size between the ex vivo model and a live pig. However, lowering the starting inoculum by multiple logs ($10^4$ EID$_{50}$ units/explant) yields similar infectious titers and growth kinetics (data not shown).
This study confirms that recently emerged H7N9 viruses are capable of infecting pigs, consistent with the work of others (11, 18). This significantly increases the risk associated with H7N9 viruses and their potential for acquisition of mutations or gene segments that could enhance transmissibility in mammals. Recent confirmed human infections in July support the idea that these viruses are still at large in the field (4), but currently, no systematic swine H7N9 influenza surveillance is performed in Northern or Eastern China. The data from this study validates the swine explant model for screening the replication potential of H7N9 viruses in porcine tissues, and more broadly, indicates this model will be valuable for screening the host range and pathogenicity of emerging influenza viruses before proceeding into live animal models.

Acknowledgements

This work was supported by Contract No. HHSN266200700005C from the National Institute of Allergy and Infectious Disease, National Institutes of Health, Department of Health and Human Services, and by the American Lebanese Syrian Associated Charities (ALSAC). We thank Shyamli Basu Roy, Alex Fedinec, Charles Leffler and Elena Parfenova for providing animals for these studies, Renee Chan for her expert advice in explant preparation, and Stephanie Sonnberg, Kimberly Friedman and James Knowles for assistance in preparing the manuscript.
FIGURE LEGENDS

FIG 1 Replication kinetics of human H7N9 viruses in swine respiratory explants.

Respiratory tissue explants from (A) trachea or (B) lungs were inoculated with $10^6$ EID$_{50}$ units/explant. At the indicated time points, virus shed from the explants was titrated in MDCK cells and presented as $\log_{10}$TCID$_{50}$/ml. Error bars indicate SD of triplicate (H7N9) viruses or duplicate (H2N2 or H2N3 controls) explants. ** p < 0.01; *** p < 0.001 (Two-way ANOVA) as compared to H2N3 swine control virus.

FIG 2 Immunohistochemical analysis of swine respiratory explants inoculated with human H7N9 viruses.

Tracheal (A, C, E, G, I) and lung (B, D, F, H, J) explants at 48 or 72 hpi inoculated with human H7N9 Anhui/1 (A, B), Shang/1 (C, D), and Shang/2 (E, F), positive control Swine/MO (G, H) or negative control Duck/NJ (I, J), influenza viruses were analyzed. Tissues were fixed and stained for influenza A virus nucleoprotein (brown) and imaged at 10x (lung) or 20x (trachea) objective magnification. In tracheal explants, influenza A viral antigen-positive cells (black arrows) were diffusely spread in tracheal epithelium inoculated with H7N9 viruses (A, C) and swine virus control (G), and as a multiple foci in influenza Shang/2 inoculated samples (E). Antigen positive cells were also found as a continuous line in bronchial epithelium (red arrows) inoculated with H7N9 viruses (B, D, F) and swine virus control (H), and as single alveolar cells (B, D, H) in lung explants. Antigen positive cells were limited to bronchial epithelium in lung explants inoculated with Shang/2 (F).
References


FIG 1 Replication kinetics of human H7N9 viruses in swine respiratory explants. Respiratory tissue explants from (A) trachea or (B) lungs were inoculated with $10^6$ EID$_{50}$ units/explant. At the indicated time points, virus shed from the explants was titrated in MDCK cells and presented as log$_{10}$ TCID$_{50}$/ml. Error bars indicate SD of triplicate (H7N9) viruses or duplicate (H2N2 or H2N3 controls) explants. ** p < 0.01; *** p < 0.001 (Two-way ANOVA) as compared to H2N3 swine control virus.
FIG 2 Immunohistochemical analysis of swine respiratory explants inoculated with human H7N9 viruses. Tracheal (A, C, E, G, I) and lung (B, D, F, H, J) explants at 48 or 72 hpi inoculated with human H7N9 Anhui/1 (A, B), Shang/1 (C, D), and Shang/2 (E, F), positive control Swine/MO (G, H) or negative control Duck/NJ (I, J), influenza viruses were analyzed. Tissues were fixed and stained for influenza A virus nucleoprotein (brown) and imaged at 10x (lung) or 20x (trachea) objective magnification. In tracheal explants, influenza A viral antigen-positive cells (black arrows) were diffusely spread in tracheal epithelium inoculated with H7N9 viruses (A, C) and swine virus control (G), and as multiple foci in influenza Shang/2 inoculated samples (E). Antigen positive cells were also found as a continuous line in bronchial epithelium (red arrows) inoculated with H7N9 viruses (B, D, F) and swine virus control (H), and as single alveolar cells (B, D, H) in lung explants. Antigen positive cells were limited to bronchial epithelium in lung explants inoculated with Shang/2 (F).