RESEARCH REPORT

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PUBLIC HEALTH/WORKER SAFETY

Title: Adaptation of a human seasonal H3 influenza A virus to efficiently infect and replicate in the swine host, **NPB #16-129**

Investigator: Dr. Amy L. Vincent

Institution: USDA, ARS, National Animal Disease Center

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Industry Summary

Influenza is an important respiratory disease of pigs, resulting in substantial economic burden to swine producers every year. Pigs were purported to be mixing vessels for influenza A viruses (IAV) due to their susceptibility to infection with IAV from different species and a potential role in generating novel reassorted viruses. This resulted in a biased view of pigs and swine agriculture as the main source of human pandemic viruses. Though there is the potential for swine viruses to be transmitted to humans, like the 2009 H1N1 pandemic, there is also evidence that human seasonal viruses frequently spillover into pigs and have a major impact in IAV diversity in swine. However, changes to the human viruses appear to be required for human-origin viruses to infect and subsequently become transmissible from pig to pig to be maintained in swine populations. In 2012, a new spillover of a human seasonal H3 IAV was recognized in the U.S. that most likely occurred in the winter of 2010-2011. This H3N1 swine virus had a mixture of swine and human IAV gene segments from at least 3 source viruses. To investigate the underlying factors that allowed this human to swine spillover, we generated viruses from a putative human H3N2 parental virus and the swine H3N1, and swapped surface genes, hemagglutinin (HA) and neuraminidase (NA), and other virus genes, in an attempt to recreate the virus detected in nature. Our results showed that although the HA was crucial for the infectivity in pigs and swine tissue, the adaptation of these novel H3 viruses to swine depended on multiple genes. The swine-adapted HA alone was not sufficient to confer the full growth potential seen for the field isolates. These findings provide important information about the requirements for a human virus to adapt in swine and can be used to monitor swine surveillance data for viruses that have adaptation signatures similar to these novel human-like viruses. Rapid identification allows pork producers and veterinarians to implement more effective control measures to avoid establishment of additional novel human viruses before they become fully adapted and widespread. By reducing the IAV burden in the swine population, the public health perception, consumer confidence, and trust by trade-partners may be improved, and, importantly, reduce the economic cost of IAV to producers and the swine industry.

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For more information contact:

National Pork Board • PO Box 9114 • Des Moines, IA 50306 USA • 800-456-7675 • Fax: 515-223-2646 • pork.org

Amy.vincent@ars.usda.gov

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Scientific Abstract
The current diversity of influenza A viruses (IAV) circulating in swine is largely a consequence of human-to-swine transmission events and subsequent evolution in pigs. However, little is known about the requirements for human IAVs to transmit to and subsequently adapt in pigs. Novel human-like H3 viruses were detected in swine herds in the USA in 2012 and have continued to circulate and evolve in swine. Reverse genetics (rg)-generated reassortants between a human-like H3N1 isolated from swine and a seasonal human H3N2 virus with common HA ancestry were evaluated by *in vitro* models to understand the contributions of individual gene segments on the ability of these viruses to infect pigs. Swine-adapted human-like H3 (hu-H3) demonstrated abundant attachment to epithelial cells from upper-, mid- and lower- swine respiratory tract tissues by virus histochemistry, while the seasonal human virus bound to fewer cells. Kinetics of virus growth in porcine intestinal epithelial cells (SD-PJEC) and in *ex-vivo* porcine trachea explants was significantly reduced by replacing the swine hu-H3 with the human seasonal H3, indicating the swine-adapted hu-like H3 was important for binding and entry in swine cells. The swine-adapted H3 with human seasonal internal genes grew efficiently at 33°C, but had decreased growth at 40°C, the temperature representing the lower respiratory tract of growing pigs. Although the swine adapted H3 was crucial for the infectivity in pigs and swine tissue, these results suggest that the adaptation of these novel H3 viruses to swine was multigenic since the swine-adapted HA alone was not sufficient to confer the full phenotype of the wild-type H3N1 parental swine virus onto the putative ancestral human H3N2 virus.

Introduction

Influenza is caused by a zoonotic pathogen that infects many species of birds and mammals, including pigs, horses, dogs, and humans (1). Influenza A viruses (IAV) are enveloped, with a single stranded segmented RNA genome, containing 8 different gene segments (2). The envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA), provide the subtypes to IAVs, are involved with viral binding and growth, and are the main targets for host immunity (2). The influenza HA recognizes sialic acid (SA) receptors on the surface of host epithelial cells with different receptor specificity: human viruses preferably bind to SA linked to galactose by α2,6 linkages and avian influenza viruses preferentially recognize α2,3-linked SA (3-5). Pigs possess both α2,6- and α2,3-galactose linked SA receptors in their respiratory tract: while α2,6-linked SA receptors are more abundant in the upper respiratory tract, there is a gradual increase in expression of SA α2,3-galactose linked SA receptors towards the lower respiratory tract (6). Swine and human adapted IAV tend to have overlapping glycan-binding properties with a α2,6-linked SA preference. Thus, swine have the possibility to serve as intermediary hosts in the evolution of influenza viruses with the potential for generation of pandemic viruses, as highlighted by the 2009 pandemic (7).

IAV endemic in swine are of three subtypes: H1N1, H1N2, and H3N2 (8), similar to those that circulate in humans. However, at least ten distinct H1 and H3 phylogenetic clades within those subtypes co-circulate in North American swine populations (8) with dramatic evolution following the introduction of a triple reassortant virus containing avian-, swine-, and human-origin gene segments (8, 9). As this example demonstrates, inter-species transmission of IAV has been documented and the bi-directional transmission between human and swine IAV contributes to the many genetic and antigenic distinct viruses circulating in pigs worldwide despite swine and human viruses being largely species-specific. Although human-to-swine transmission events are relatively common (10), wholly human seasonal viruses do not usually become established in pigs, rather individual gene segments become incorporated into reassortant viruses and when the human HA is maintained, it often has

substantial genetic differences from the seeding human strains by the time it is detected in swine (10), suggesting that adaptation to the swine host is required for the persistence of such viruses.

Since 2012, novel H3N2 and H3N1 viruses were identified through the United States Department of Agriculture (USDA) swine IAV surveillance system, containing HA genes from contemporary human seasonal H3N2 that circulated in 2011-2012 (11). These human-like viruses have been detected from a minimum of three generations of reassortment events, each generating a virus with a unique genome constellation: the initial case contained human H3, human N2, and internal genes from the 2009 pandemic H1N1 (H1N1pdm09); in the second generation virus, the N2 was replaced by classical swine N1; and then evolved to a third generation of reassortants with N2 from the swine 2002 lineage, M gene from H1N1pdm09, and remaining internal genes from the triple reassortant internal gene (TRIG) constellation (11). These human-like viruses were shown to be genetically and antigenically distinct from swine H3 viruses currently circulating in the U.S. and from vaccine strains used in pigs, as well as human seasonal H3 circulating globally (11). Additionally, our group has demonstrated that these human-like viruses are fully adapted to pigs, resulting in pathologic lesions and transmission to indirect contacts, while the prototypic human precursor was unable to efficiently infect or transmit in pigs (11). Further, these data suggest that the modifications in the HA gene from the swine viruses was essential for the adaptation of these human-like viruses to pigs, although the pandemic lineage backbone appeared to play a role (11).

Although the molecular basis for IAV host-range restriction is not fully understood, some mechanisms have been shown to contribute to viral host specificity, such as the compatibility of the HA to the host SA linkage or the presence of glycan modifications on the HA protein (4, 12, 13). Susceptibility of different IAV to the antiviral activity of surfactant protein-D (SP-D) increases as the number of glycosylation sites on the virus HA increases (14). Amino acid substitutions in or near the receptor binding site in the HA can change the receptor preference from α2,3 to α2,6-SA linkages (5). Additionally, amino acid 627 in the PB2 gene has been associated to differences in virus polymerase efficiency at different temperatures (15). Understanding these mechanisms of adaptation to the swine species will allow for more rapid identification of human viruses transmitted to swine with potential to maintain infection and transmission and to implement more effective control measures to avoid establishment of additional novel human viruses in the future.

Objectives

Objective 1. Investigate viral factors associated with adaptation to the swine host, such as viral gene segments, amino acid substitutions, or glycosylation sites on the HA protein.

Objective 2. Evaluate host factors associated with viral attachment or replication (body temperature and receptor distribution in the respiratory tract using in vitro models to recapitulate the swine host.

Material and Methods

Viruses: Eight viruses were generated by reverse genetics (rg) as previously described (16) using a human-like H3N1 virus (A/Swine/Missouri/A01410819/2014; Sw/MO/14) detected through the USDA IAV swine surveillance system and a human H3N2 virus (A/Victoria/361/2011; A/VIC/11) with similar HA ancestry as parental viruses. We previously rescued these viruses to use in an *in vivo* study (11). (Table 1).

Table 1. Genetic composition of reassortant viruses generated using A/Victoria/361/2011 (A/VIC/11) and A/Swine/Missouri/ A01410819/2014 (Sw/MO/14).

Virus	HA gene origin	NA gene origin	Internal genes origin
Sw/MO/14rg	Sw/MO/14	Sw/MO/14	Sw/MO/14
VIC11-HA/NA	A/VIC/11	A/VIC/11	Sw/MO/14
VIC11-HA	A/VIC/11	Sw/MO/14	Sw/MO/14
VIC11-NA	Sw/MO/14	A/VIC/11	Sw/MO/14
A/VIC/11rg	A/VIC/11	A/VIC/11	A/VIC/11
MO14-HA/NA	Sw/MO/14	Sw/MO/14	A/VIC/11
MO14-HA	Sw/MO/14	A/VIC/11	A/VIC/11
MO14-NA	A/VIC/11	Sw/MO/14	A/VIC/11

HA= hemagglutinin; NA= neuraminidase

Virus attachment and entry: To evaluate the role of the HA and NA on viral attachment and entry, two permissive cell lines, Madin-Darby canine kidney (MDCK) cells and porcine jejunal epithelial cells (SD-PJEC, (17)), were infected in 96-well plates at a multiplicity of infection (MOI) of 10 in duplicates with each of the rg viruses. After 12h incubation at 37°C in 5% CO2, plates were fixed with 4% phosphate buffered formalin and stained using swine hyperimmune serum against the respective parental virus and fluorescein isothiocyanate (FITC)-labeled anti-swine IgG (Southern Biotech, Birmingham, AL). To quantify the entry of the different reassortant viruses into the cells, MDCK and SD-PJEC cells in 12 well plates were infected at an MOI of 5 in duplicates with each of the reassortant viruses. After 8h incubation at 37°C in 5% CO2, cells were harvested, fixed, permeabilized and stained using a mouse anti-Influenza A virus monoclonal antibody (HB65, ATCC, Manassas, VA) and R-phycoerythrin (PE) conjugated anti-mouse IgG2a antibody (Southern Biotech, Birmingham, AL).

Virus attachment in swine respiratory tissue: To evaluate the tissue tropism of the reassortant viruses in the upper and lower respiratory tract of pigs, concentrated rg viruses were inactivated by dialysis against 1% formalin and used for virus histochemistry as described previously (18). Deparaffinized and rehydrated formalin-fixed and paraffin-embedded (FFPE) respiratory tissues (nasal turbinate, tonsil, trachea, and lung) obtained from naïve 5 week-old pigs were incubated with the FITC-labeled influenza viruses overnight at 4°C. Virus-attachment was then detected with peroxidase-labeled anti-FITC antibody (Dako, Glostrup, Denmark), using the tyramide signal amplification (TSA) biotin system (PerkinElmer, Waltham, MA).

Growth kinetics in swine cells and trachea explants: The role of the surface glycoproteins and internal gene cassettes on viral kinetics was analyzed by inoculating MDCK and SD-PJEC cells at MOI of 0.01. Supernatants were collected at 12, 24, 48, and 72 h after incubation at 33, 37 or 40 °C in 5% CO2, and virus titers were determined as previously described (19). To compare kinetics in swine trachea explants, tracheas were obtained from naïve 5 week-old pigs and 8 mm punches were cultivated in 48-well plates in an air-liquid interface as previously described (20). After 18 h incubation at 37°C in 5% CO2, explants were inoculated with 10^6 50% tissue culture infective dose ($TCID_{50}$)/mL of each virus in a volume of 300 µl.

Polymerase activity assay: A minigenome reconstitution assay was used to assess polymerase activity as previously described (21), using the BioLux Gaussia luciferase assay kit (New England Biolabs, Ipswich, MA) and the Phospha-Light secreted alkaline phosphatase reporter gene assay system (Thermo Fisher Scientific, Waltham, MA) according to the manufacturers' protocols. 293T cells were transfected with the minimum number of genes required for viral transcription and replication. Additionally, an influenza virus reporter plasmid, encoding the *Gaussia* luciferase gene flanked by the 5' and 3' of the non-translated regions of the NS gene, was co-transfected and

luciferase enzymatic activity is quantified to serve as an indirect measure of viral replication. At 24 hours post transfection, the supernatant was harvested and assayed for luciferase activity. Polymerase activities were determined from two independent experiments, each performed with three biological replicates and assayed in triplicate (means ± standard error of the mean).

Reduction of infection by porcine Surfactant Protein D: No SP-D was commercially available, so the SP-D had to be custom synthesized (Life Technologies). A commercially available mammalian expression vector was employed to clone and express the swine surfactant protein D tagged with a histidine peptide for purification purposes (Genbank protein accession number NP_999275). Expi293 cells were transfected and the recombinant SP-D was detected in the supernatant as assayed by Coomasie stain and detected using a his-tag specific antibody. A plasmid encoding GFP with a his-tag was included as a control to assess transfection efficiency and expression levels. To compare the susceptibility of the different viruses to porcine SP-D, various concentrations of recombinant SP-D were incubated with each respective virus and then transferred to MDCK cells and incubated for 24h at 37°C as previously described (14). Then, cells were stained by immunocytochemistry as previously described (19).

Results

Objective 1. Virus attachment and entry. The human-like virus (Sw/MO/14) bound abundantly to epithelial cells of the nasal turbinates, trachea, bronchioles, and alveoli of pigs as shown by virus histochemistry (Fig. 1). In contrast, minimal virus attachment was observed with the human seasonal virus (A/VIC/11) (Fig. 1).

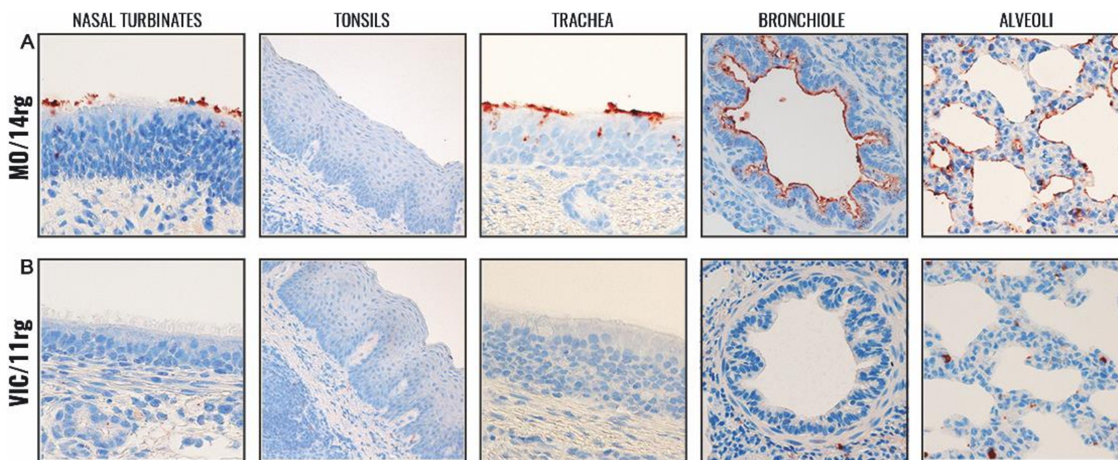
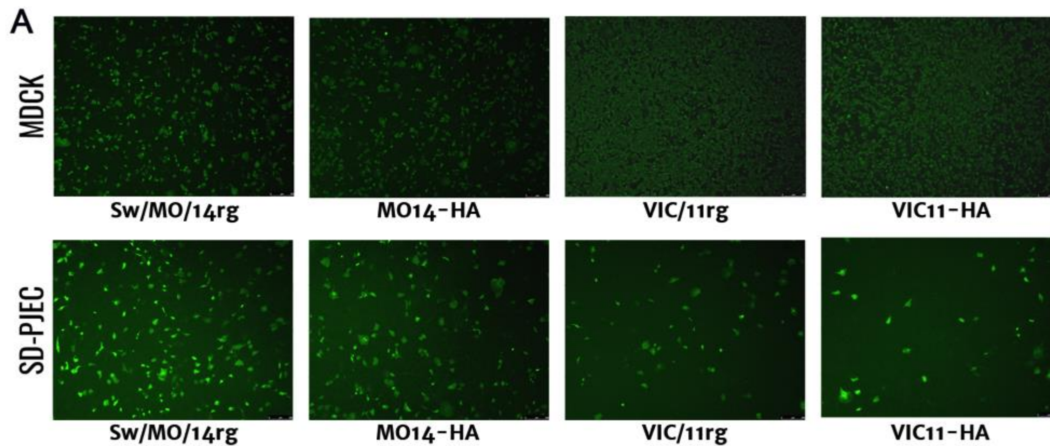


Figure 1. Attachment profile of (A) A/Swine/Missouri/A01410819/2014 (MO/14rg) and (B) A/Victoria/361/2011 (VIC/11rg) to formalin-fixed, paraffin-embedded swine respiratory tissue. Peroxidase-labeled biotin system with 3-Amino-9-ethylcarbazole (AEC) substrate and hematoxylin counterstain, 200-400 X.

Virus entry in MDCK cells was evident with viruses expressing both the swine-adapted (Sw/MO/14) and human seasonal (A/VIC/11) HA (Fig. 2), with a statistically significant higher number of positive cells with viruses expressing the A/VIC/11 HA compared to viruses that expressed the sw/MO/14 HA (Fig. 2B). However, when virus entry in the swine cell line (SD-PJEC) was examined, a statistically significant greater number of positive cells was observed with viruses that expressed the sw/MO/14 HA (Fig. 2B), indicating the sw/MO/14 HA conferred greater binding and entry into swine cells.



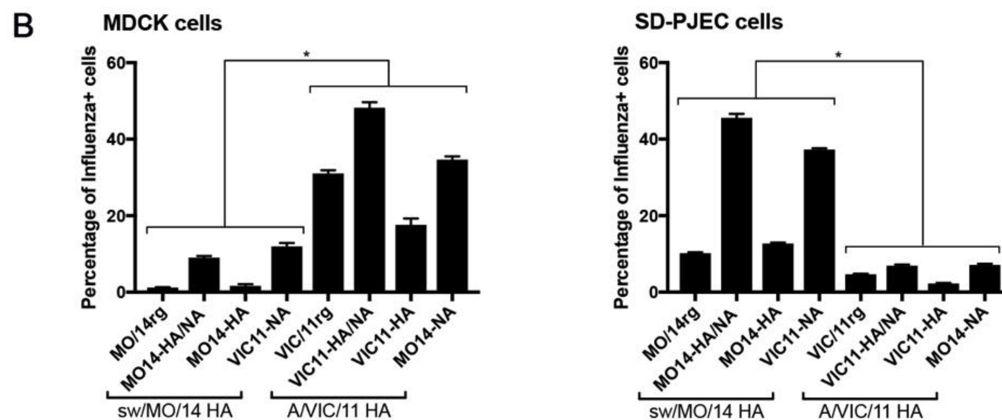
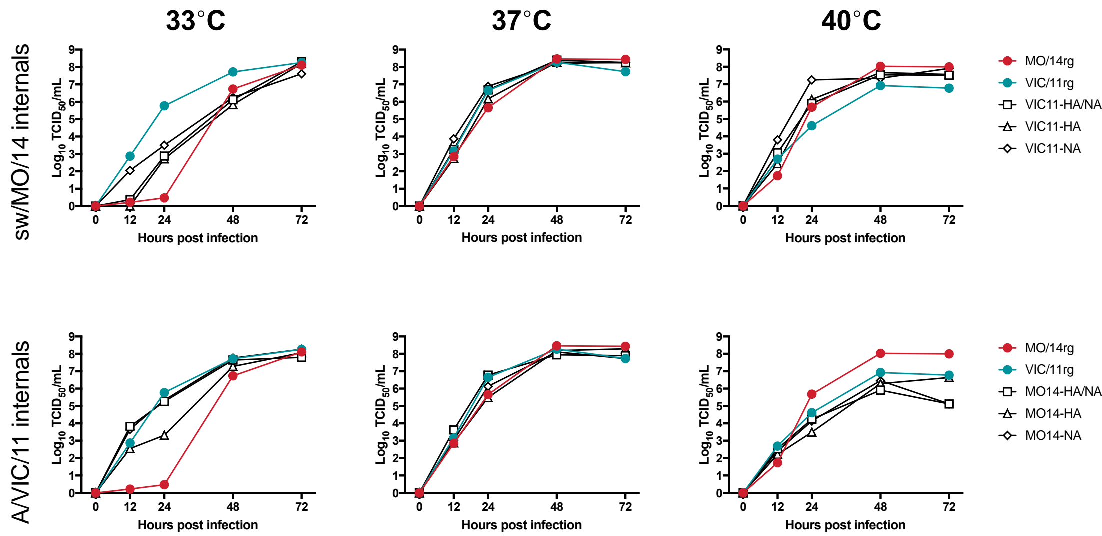


Figure 2. Entry of reassortant viruses into MDCK and SD-PJEC cells. Virus infected cells were detected by immunofluorescence at 12 hrs post infection 5X (A) or by FACS on a flow cytometer at 8hrs post infection (B) in MDCK and SD-PJEC cells.

Growth kinetics. The growth kinetics in MDCK and SD-PJEC cells at 33°, 37° and 40°C of the reassortant viruses are shown in Figure 3. All reassortants increased in titer similarly in MDCK cells at 37°C. However, different growth kinetics were observed in the swine SD-PJEC cells: switching the MO14 HA gene with the human seasonal HA gene caused a significant loss in growth efficiency compared to the viruses with the MO14. The virus with the MO14 HA gene and VIC11 N2 gene showed efficient growth similar to the sw/MO/14rg whole virus, suggesting that the N1 gene did not have a major role for the growth efficiency of the human-like viruses. Viruses with the MO14 HA gene and VIC11 internal genes showed only mildly increased growth compared to the VIC11 whole virus, not reaching the full growth efficiency of the whole virus sw/MO/14. At 33°C, viruses with the VIC11 internal genes replicated more efficiently than viruses with the MO14 internal genes in both cell lines, although viruses with the VIC11 HA gene did not replicate efficiently in the swine cell line compared to MDCK. In contrast, the opposite was observed at 40°C, as viruses with VIC11 internal genes had lower growth compared to whole virus sw/MO/14, and the lower efficiency was also observed for viruses with the VIC11 HA gene in the swine cell line.



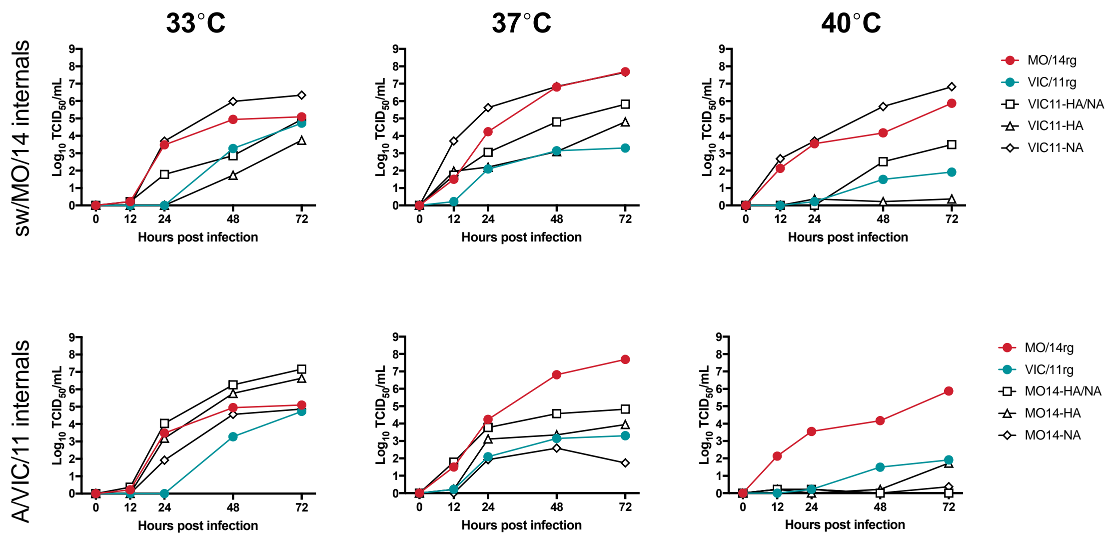


Figure 3. Growth kinetics of reassortant viruses in (A) MDCK and (B) SD-PJEC at 33°, 37°, and 40°C. Cells were infected at MOI = 0.01 and supernatants were collected at 12, 24, 48, and 72 h post-infection.

The growth kinetics of the panel of reassortant viruses were also assessed in swine trachea explants at 37°C. Overall, most viruses with the swine-adapted HA (MO14) showed increased growth efficiency in swine *ex-vivo* trachea explants compared to the viruses with human seasonal HA (VIC11; Fig. 4).

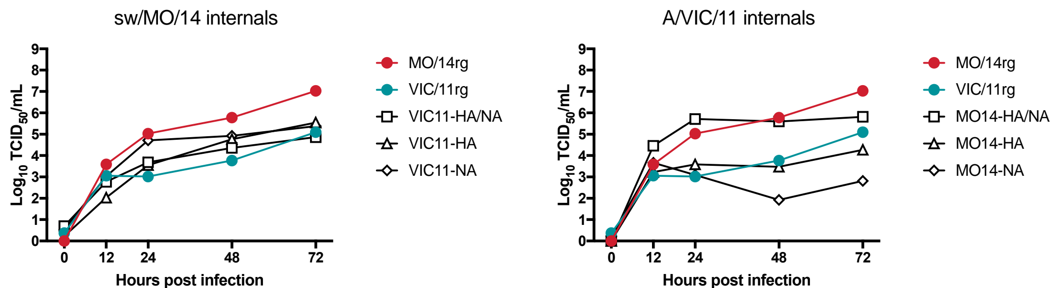


Figure 4. Growth kinetics of reassortant viruses in trachea explants at 37°C. Explants were infected with $3x10^5$ TCID50 and supernatants were collected at 12, 24, 48, and 72h post-infection.

Polymerase activity. To assess the role of the polymerase genes acting in concert as the viral replicase in the growth kinetics, a minigenome assay was performed using the internal genes (PB2, PB1, PA and NP) of MO/14 or VIC/11 and a reporter gene (luciferase) to indicate levels of RNA replication. Internal genes from VIC/11 replicated to significantly higher levels at all three temperatures tested (Fig. 5). Consistent with delayed growth kinetics at 33°C of the viruses with MO/14 internal genes in MDCK and SD-PJEC cells, the MO/14 replicase genes were least efficient at 33°C and replicated to increasingly higher levels at 37 and 40°C in the 293T cells. The replicase genes of a well-described swine lineage virus (A/turkey/Ohio/313053/2004) was used as a control, and replicated to comparable levels when compared to MO/14 at 40°C, but MO/14 replicated to significantly higher levels at 33 and 37°C.

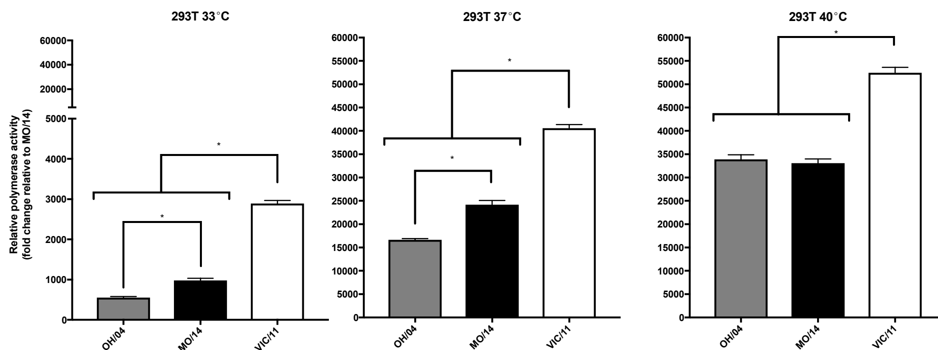


Figure 5. Viral ribonucleoprotein reconstitution assay to study the polymerase activities at 33°, 37°, and 40°C in 293T cells.

Reduction of infection by porcine Surfactant Protein D. The swine surfactant protein D was cloned and expressed with a histidine peptide tag for purification purposes (Genbank protein accession number NP_999275). The custom expressed recombinant SP-D was obtained from the commercial company after confirmation with Coomasie stain and western blot staining with a his-tag specific antibody. Preliminary tests using initial aliquots with the purified SP-D protein indicate that the

methodological approach will have to be optimized to improve the signal of positively infected cells and assess susceptibility of MO/14 and VIC/11 to SP-D.

Discussion

The ecology of swine influenza A viruses (IAV) is complex, with different strains and subtypes co-circulating in pigs in the United States. The current diversity is largely a consequence of human-to-swine transmission events and subsequent evolution (10). Despite the importance of these reverse zoonotic episodes, little is known about the circumstances under which human IAVs transmit and become adapted to swine. Although human-origin viruses and/or viral segments sporadically become endemic in pigs, the lineages that have become established, such as the triple reassortant internal gene (TRIG) constellation, have contributed enormously to the viral evolution and the current problems producers are faced with in terms of effective vaccination and control strategies (8, 10). More recently, in 2014-15, human-like H3 viruses were detected in swine herds in the U.S.A. (11). The most closely related ancestor were human seasonal viruses from the 2010-11 flu season, but the swine viruses already exhibited many genetic changes compared to the human precursors, most notably in the HA gene and in reassorted internal gene constellations. Here, we found that the adaptation of novel human H3 viruses to pigs was a multigenic trait and that the swine-adapted HA alone was not sufficient to confer the full phenotype of the wild-type parental virus.

By using reassortant viruses generated by reverse genetics, the surface genes (HA and/or NA) were swapped onto the internal genes of a swine human-like virus (MO/14) and a human seasonal H3N2 virus (VIC/11). This enabled us to investigate the role of surface and internal genes on the adaptation of 2010-lineage of human-like H3 viruses to pigs. The HA was indicated to be the limiting factor for the adaptation of these viruses to pigs, and we showed that viruses with the swine-adapted HA gene (MO14) showed more efficient binding and entry into swine tissue and swine cells. However, the MO14 HA alone was not enough to recover the growth kinetic properties of the human internal genes, the swine virus sourced internal genes were necessary for the full efficiency seen in swine tissue and in swine cells.

The basal temperature at the primary replication site can interfere with virus polymerase efficiency. This difference was attributed to amino acid 627 in the PB2 gene (15). While the optimal replication temperature of avian viruses is close to 40°C, the temperature of the avian intestinal tract, the optimal temperature of human IAV is 33 to 34°C, similar to the human upper respiratory tract. The temperature of the upper respiratory tract of pigs is approximately 37°C and even higher in the lower respiratory tract. Consistent with host body temperature, switching the sw/MO14 adapted swine genes back to the VIC11 human virus internal genes increased growth kinetics at 33°C both in MDCK and SD-PJEC. However, these reassortants had decreased replication at 40°C. The internal genes of sw/MO/14 exhibited improved growth kinetics at 37° and 40°C, compared to growth kinetics at at 33°C. The minigenome assay, an assay that solely examines the RNA replication of the viral ribonucleoprotein complex, was consistent with the growth kinetics data, demonstrating that the VIC/11 genes replicate better at 33°C and reassortant viruses comprised of VIC/11 internal genes grew at faster rates at 33°C.

The VIC/11 internal genes are of the H1N1pdm09 lineage, which has been shown to efficiently replicate in both the upper and the lower respiratory tracts of pigs (22). However, viruses with the VIC/11 HA did not replicate well in the swine cell line at either temperature, likely a result of the lower attachment and entry. Additional studies comparing MO/14 and VIC/11 susceptibility to swine surfactant protein D may provide another factor associated in host-specific restrictions. All together, these results indicate that the swine-adapted HA gene was essential for optimal growth in swine cells by its efficient receptor binding and entry properties. Later stages in the virus lifecycle, such as RNA replication by the viral replicase contributed to adaptation, although this was not observed in the non-

swine cells (293T) under the conditions we tested. Additional polymerase activity assays in swine cells are necessary to draw conclusions about the role of these genes to the overall adapation in swine. Understanding these mechanisms of IAV adaptation from human to swine hosts will allow for more rapid identification of human viruses transmitted to swine with potential to maintain infection and transmission. Early detection of these human spillover viruses will enable implementation of more effective control measures to avoid establishment of additional novel human viruses in the future.

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