STUDYING ADAPTATION OF HUMAN ORIGIN H3N2 VIRUSES IN SWINE

by

JONGSUK MO

(Under the Direction of Daniela S. Rajao)

ABSTRACT

Influenza A viruses (IAV) cause respiratory disease in many host species, including humans and pigs. The spillover of IAV between swine and humans poses a challenge for the swine industry and public health. IAV have a segmented genome which consists of 8 negative-sense RNA gene segments that encode 10 major proteins and several strain-specific minor proteins. Hemagglutinin (HA), the major surface glycoprotein, is a determining factor for host specificity. IAVs carrying a triple reassortant internal gene (TRIG) constellation, containing genes from human-, swine- and avian-origin, emerged in swine in North America in the late 90s, and has contributed to the establishment of human-origin IAVs in pigs and increased viral diversity. While there is frequent evidence of human IAVs being introduced into swine populations, the molecular determinants driving the adaptation to pigs is not well understood. By using a reassortant recombinant IAV containing surface genes of human-origin IAV, we showed the virus was able to replicate and transmit in pigs. Next, we studied the viral evolution by performing serial passages using differentiated swine cells as an *in vitro* infection model. Sequencing and variant analysis from the *in vivo* and *in vitro* studies revealed several mutations arose after replication in pigs or swine cells. All mutations were on the head of the HA. We

reconstructed the mutant viruses via reverse genetics and showed they replicate more efficiently in swine cells compared to viruses containing the wild type human-origin IAV HA. Overall, our results show mutations quickly arise after replication of human-origin IAV HA in swine and result in improved fitness.

INDEX WORDS: Influenza A virus, Hemagglutinin, Viral evolution, Host specificity, Triple reassortant internal gene (TRIG), Next generation sequencing, Reverse Genetics, Variant Analysis, Viral growth kinetics, Primary swine epithelial cell, Cell differentiation, Serial passage

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DEDICATION

I dedicate this work to my wonderful family, In-Pil Mo, my father, and colleague who have inspired me and provided valuable advice in following in his footsteps as a fellow veterinarian and virologist, my mother Ki-Ok Park, who has always held my hand and supported me with unconditional love and wisdom. And lastly but not least, my brother Jong-Seo Mo, my closet colleague and brother-in-arms pursuing the joy of science together.

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CHAPTER 1

INTRODUCTION

Influenza A viruses (IAV) are enveloped viruses that cause respiratory diseases in several species, including humans and pigs. IAVs belong to the *Orthomyxoviridae* family, and have a segmented genome, comprising eight negative-sense, single-stranded viral RNA gene segments [1]. IAVs have a wide host range and the ability to adapt and cross between species [2], and cases of IAV crossing between humans, swine and avian species have been shown in numerous studies [3-5].

Hemagglutinin (HA) is a major surface glycoprotein of IAV and plays an important role in host specificity and mediating entry into the host cell. The initial infection stages of IAV involves the binding of HA to sialic acids (SA) via the receptor binding sites (RBS). There are two forms of linkages that the HA utilizes during the binding process: SA-α-2,3 linkage, which is commonly expressed in avian species, and SA-α-2,6 linkage, which is prominent in human [6]. Since the first stages of IAV infection involve binding of HA to the host cell membrane, the host-specificity of the HA is partially defined by such ability to bind to these receptors [7]. The HA is a highly variable protein. Mutations on the HA can alter receptor binding profiles, and some changes may be able to promote host-jumping [5]. For example, changes in amino acid position 226 (H3 numbering) in H9N2 and H3N2 IAVs have been shown to affect SA binding preference [8]. Additionally, six to seven positions near the RBS, including 145,

155, 156, 158, 159 and 189, were shown to affect antigenicity in human and swine H3N2 IAV [5], and could potentially also alter host tropism, although evidence is still lacking.

IAVs circulate globally in pigs, with the major subtypes including H1N1, H3N2, and H1N2. Significant genetic and antigenic diversity exists which reflects the high variability of IAV [9]. The diversity of viruses circulating in North American swine has increased significantly after the introduction of a virus containing a triple-reassortment internal gene (TRIG) constellation in the late 90s [10]. The TRIG cassette contains internal genes derived from swine (matrix, non-structural and nucleoprotein), human (polymerase basic 1 -PB1), and avian (polymerase acidic and PB2) influenza viruses. Most of the swine strains in the U.S. contain some (or most) internal genes from this lineage, with most of the variation concentrated on the HA and NA. The TRIG cassette is unique as it is capable of stably incorporating HAs and NAs from various origins without adversely affecting the fitness of the virus [11]. Importantly, the TRIG cassette is one of the lineages endemic in swine that reassorted to generate the pandemic H1N1 (H1N1pdm09) [12].

Interspecies transmission between humans and swine are relatively common and can contribute to the generation of various novel lineages and to increased diversity of viruses circulating, particularly in swine [5, 13]. Surveillance of swine IAV showed separate cases of human-origin IAV that were introduced into the swine populations globally, most notably seasonal H1 and H3 IAV, and the 2009 pandemic H1N1 virus (H1N1pdm09). Even after a decade since the pandemic, H1N1pdm09 viruses are continually being transmitted from humans to pigs globally [3]. In the U.S., a recent spillover of human H3N2 viruses led to the establishment of a new lineage, which is now

one of the most dominant swine H3N2 lineages in the country [13, 14]. In fact, most viruses that are currently endemic in pigs in the U.S. are from human-origin. Thus, studying these human-to-swine spillovers and how the viruses evolve between species is of high importance, not only to control economic losses in the swine industry but also for public health.

Research objective and aims

While there is ample evidence of human-origin gene segments persisting in the swine IAV population, the mechanisms enabling the adaptation of human IAV (or their gene segments) to swine are not well known. Most human-origin surface gene segments that persisted in swine globally have undergone significant change [9, 15] suggesting that molecular changes have to occur in the HA to enable human IAVs to adapt to swine. These mutations could potentially affect the receptor binding profile and induce conformational change to bind to receptors that are more abundant in the swine respiratory tract. The main goal of this dissertation is to determine the molecular mechanisms that enable adaptation of human origin IAV in swine by using *in vivo* and *in vitro* infection models. For this purpose, we have used a reassortant recombinant H3N2 virus containing human-origin IAV HA and NA with similar ancestry to the 2010 H3N2 lineage that currently is endemic in U.S. swine. The first part of the dissertation explores the molecular changes that may enable a human-origin virus to successfully transmit and replicate in pigs, with emphasis on the HA and NA. We have used sequencing, variant analysis, and structure modeling to plot the locations of the amino acid mutations. We also tested how the molecular changes affected the replication profile of the strain by using differentiated swine tracheal cells. In the second part of the dissertation, we have

simulated long-term evolution by sequentially passing the virus in differentiated swine tracheal cells to evaluate changes that would enable the successful adaptation in swine. The specific aims of the dissertation are as follows:

1) Evaluate influenza virus evolution after infection of swine with a human-origin H3N2 reassortant influenza virus.
    a) Evaluate replication and transmission of the strain in swine (conducted by United States Department of Agriculture-Agricultural Research Service; USDA-ARS) and study changes in viral sub-populations that emerged during the animal study
    b) Evaluate effect of the mutations that arise during replication and transmission in pigs in virus replication and fitness.
2) Evaluate effects of long-term evolution of a human-origin H3N2 reassortant influenza virus in differentiated swine cells
    a) Set up an *in vitro* infection model by using differentiated swine trachea cells
    b) Study changes in viral sub-populations that may emerge during serial passage in swine cells.
    c) Evaluate the effect of the mutations that arise during replication and passage in differentiated swine trachea cells in virus replication and fitness.

CHAPTER 2

LITERATURE REVIEW

Influenza viruses are enveloped viruses that cause respiratory diseases in several species, including humans, avian species, and pigs. They belong to the family *Orthomyxoviridae.* The family contains seven genera: *Alphainfluenzavirus, Betainfluenzavirus, Deltanfluenzavirus, Gammainfluenzavirus, Isavirus, Thogotovirus, Quaranjavirus* [16, 17]. The virus genome is segmented with a spherical or pleomorphic morphology and around 80-120nm in diameter.

Influenza A viruses (IAV) belong to the *Alphainfluenzavirus* genus, which are responsible for causing most of the epidemics in respiratory disease in humans during the 20-21th centuries [16]. IAV can infect a large number of animal species. Wild aquatic birds and waterfowl are considered the reservoirs for IAV and the source of virus to other species. Interspecies transmission between other hosts is also possible, making the ecology of IAV more complex [3, 4, 18]. IAVs contain a segmented genome with a length of around 14kb, comprising eight negative-sense, single-stranded viral RNA segments [1]. There are two surface glycoproteins, the HA and the neuraminidase (NA), which mediate entry, infection, and release, and whose antigenic properties are also used to divide IAV into subtypes. So far, 18 HA and 11 NA subtypes of IAV have been detected in nature, most of which have been detected in the wild aquatic waterfowl reservoirs [1, 19]. The HA subtypes are divided into phylogenetic groups based on

sequence similarities on the C-terminal 1/3 of the HA molecule: Group 1 consists of H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16; Group 2 consists of H3, H4, H7, H10, H14, and H15. IAVs caused 4 pandemics in the last 110 years, the pandemics of 1918 and 2009, which were caused by H1N1 viruses, and the 1957 "Asian Influenza" and 1968 "Hong Kong Influenza" pandemics, which were caused by H2N2 and H3N2 viruses, respectively.

The genus *Betainfluenzavirus* contains the species Influenza B virus (IBV), which have eight genome segments and HA and NA similar to IAV [16]. In contrast to IAV, they are classified into two distinct lineages, the B/Victoria/2/87-like and B/Yamagata/16/88-like viruses. IBVs mostly affect humans, although there have been sporadic cases in seals [19]. Compared to IAV, IBV tend to undergo antigenic change more slowly. The *Gammainfluenzavirus* genus contains the species Influenza C virus that infects mostly humans, particularly children, and occasionally swine [16]. Unlike IAV and IBV, they only have seven gene segments, with missing NA protein. Instead, a unique protein (HEF) is known to have receptor-destroying capabilities by utilizing the 9-0-acetylnueraminyl esterase [20]. Lastly, the *Deltainfluenzavirus* genus contains the species Influenza D virus [21]. They infect mostly pigs and cattle, and no human infections have been observed to date. Like Influenza C viruses, members of the species contain seven RNA segments.

Influenza A virus genome organization

Each of the eight segments of IAV perform a specific function for viral replication. Segment 1 (PB2), Segment 2 (PB1) and Segment 3 (PA) encode viral

proteins which comprise the viral polymerase. Segment 4 (HA) encodes the hemagglutinin, which is a glycoprotein of the viral envelope with a crucial role in host receptor binding and membrane fusion [1, 2]. Segment 5 (NP) encodes the viral nucleoprotein, which is an RNA binding protein that regulates nuclear import. Segment 6 (NA) encodes the neuraminidase, which contains sialidase activity, promoting cell release [22]. Segment 7 (M) encodes 2 proteins, M1 which comprises the matrix protein, and M2 which comprises the ion channel and promotes virus uncoating and assembly once inside the host cell. Segment 8 (NS) encodes the non-structural proteins, which regulate host gene expression and nuclear export of RNA [1].

Segment 1 (2341 nts): Polymerase basic 2 (PB2)

Segment 1 encodes the PB2, which is a part of the polymerase complex and interacts directly with PB1 to promote viral transcription and replication [1, 23]. The PB2 protein has an average length of 759 amino acids and has a role in mRNA cap recognition [1]. PB2 interacts with the NP via the C terminal region of the protein [23, 24]. The PB2`s role in host specificity is associated with amino acid position 627, in which mutations have been shown to alter tissue tropism and pathogenicity in different hosts [25, 26]. Recently, a separate gene product, PB2-S1 was shown to have a role in suppressing host immunity by altering the interferon signaling pathway by localizing to the mitochondria complex [27].

Segment 2 (2341 nts): Polymerase basic 1 (PB1, PB1-N40, PB1-F2)

The PB1 is encoded in segment 2. PB1 is also part of the IAV polymerase complex and is involved in RNA elongation and endonuclease activity [1, 23]. It has an

average length of 757 amino acids. PB1 contains overlapping regions in which vRNA, PA and PB2 can bind and interact [28]. PB1 contains highly conserved motifs among viral RNA-dependent, RNA-polymerases (RdRp), thus functioning as a catalytic subunit. The viral RdRp complex consists of PB1, PB2 and PA subunits [29, 30]. In addition to the PB1 protein, PB1-N40 and PB1-F2 have been identified [23, 31]. The role of PB1-N40 is not fully understood but it may support IAV replication [32]. Contrarily, PB1-F2 acts as a immunomodulator to increase virus replication by promoting cell death [33]. The gain of PB1-F2 function has been shown to affect virus replication in both mice and swine by modulating immune response [34]. Thus, in addition to its function as a viral polymerase, PB1 also has a role in suppressing the host immune system.

Segment 3 (2233 nts): Polymerase acidic (PA, PA-X)

PA is the last member of the viral RdRp complex and consists of 716 amino acids, encoded in segment 3 [1]. PA functions as a endonuclease which is crucial in the cap-snatching mechanism during replication [23]. PA is involved in the first stages in the assembly of the viral RdRp complex by binding to PB1 first in the cytoplasm and then migrating to the nucleus to bind PB2, completing the assembly [35]. Thus, viral ribonucleoprotein (vRNPs) complexes are formed, which consists of the viral RdRp complex, nucleoprotein (NP) and vRNA [36] Mutations in PA can lead to reduced proteolysis efficiency [37]. In addition to PA, the segment also encodes PA-X, which is known to suppress and prevent host cell gene expression by preventing host mRNA function [38]. In swine, previous work showed that the PA-X enhanced replication and transmissibility of an H1N2 by playing a role in immunosuppression and delaying proinflammatory response [39].

Segment 4 (~1700 nts): Hemagglutinin (HA)

Segment 4 (exact size depends on subtype) encodes a single open reading frame, the HA. HA is the major envelope glycoprotein of IAV and is a critical component in determining host specificity due to its role in initial binding and entry into the host cell [2]. The HA has an average length of 566 amino acids and functions as a major antigen for antibody recognition [1].

The HA protein is formed of 2 subunits, the HA1 and HA2 [40] that result from the cleavage of the precursor HA0 protein. Part of the HA1 forms the globular head of the HA, whereas the remaining parts of HA1 and the HA2 form the stalk. The HA binds via its receptor binding site (RBS) to sialic acids (SA) in the host cell containing either an α-2,3 linkage or α-2,6 linkage to the galactose. The RBS is the form of a pocket on the HA head, which allows the HA to dock and bind to SA receptors [41]. The RBS has three key structures which are involved in the receptor binding process: the 130-loop, 190-helix and the 220 loop [5]. The type of linkage (α-2,3 or α-2,6) on SA receptors alters conformation, impacting binding to the RBS. Hence, IAV evolve to preferentially bind specific types of receptors present in the host species and host-specificity is in part determined by the HA ability to bind to these receptors [7]. SA-α-2,3 conformation is dominantly expressed in avian species, particularly in the respiratory and intestinal epithelial cells. SA-α-2,6 conformation is dominantly expressed in the upper respiratory tract of humans [6]. Thus, viruses that are adapted and established in these species tend to bind preferably to these receptors. Specific amino acid substitutions on or near the RBS can change receptor-binding preference and facilitate host jumping among species [18]. Critical substitutions have been shown to affect the receptor binding profile of IAVs and

enable species crossing, such as the 226 amino acid position of avian H9 IAVs, which was shown to be flexible and capable of incorporating various residues [8, 42]. Amino acid position 226 is also critical for receptor specificity of H3 viruses [8]. Likewise, several amino acid positions in the HA of human and swine H3N2s were shown to affect antigenicity of the strains [43]. The evolution of IAVs has been consistently linked to mutations in the HA, although the rate of amino acid substitution are known to differ by species [44]. For swine, the substitution rate for H1 was 4.6×10^{-3} substitutions per site per year with majority of the mutations occurring in the head of the HA, showing substantial antigenic drift occurs in swine IAVs [45]

Segment 5 (1545 nts): Nucleoprotein (NP)

The NP is a highly conserved protein and is important to maintain integrity of the RNA and regulate nuclear import [1]. It is known to have an average size of 498 amino acids. The main function of the NP is to bind and encapsulate vRNA through its positively charged regions, as vRNAs have an abundance of negatively charged groups [46]. After binding to vRNAs, the NP promotes nuclear import by utilizing its own nuclear localization signals (NLS) and the NLS from the viral polymerase complex [23]. The nuclear import of the NP is mediated through importin-α, which then binds to karyopherin B [47, 48]. Interestingly, there is a study showing the NP gene of swine origin may be able to interact with human-origin IAV proteins [49]. The NP of the swine H1N1 was constructed as a bait to screen human cDNA prey library and was shown to interact with human proteins involved in purine biosynthesis, cytoskeleton and DNA binding.

Segment 6 (~1400 nts): Neuraminidase (NA)

The NA, the second major envelope glycoprotein, possesses sialidase enzymatic activity and is responsible for cleaving the SA from the cellular surface, enabling the release of progeny viruses from the host cell [50]. The NA is also involved in the cleavage of decoy receptors present on mucins in the respiratory system, contributing to viral infection. The NA is approximately 469 amino acids long. Similar to HA, the NA is also classified in two groups with group 1 consisting of N1, N4, N5 and N8 and group 2 consisting of N3, N6, N7, N9 [51]. The balance between the NA enzymatic activity and the HA binding avidity is important to guarantee virus stability and can also affect the host range of IAVs. The balance of activity between HA and NA was recognized as a key mechanism in the evolution of the pdm09 H1N1 [52] and essential for its respiratory transmissibility [53]. The NA is one of the prime target of antivirals against IAV, leading to successful development of NA inhibitors such as Zanamivir and Oseltamivir [51]. A study suggested the NA of swine IAVs upregulate inflammatory factors [54]. In that study, a reassortant generated between a H1N1pdm09 strain and an endemic swine H1N2 strain was able to increase macrophage inflammatory factor-2 (MIP-2) in mice, which resulted in increased pathogenicity, especially in the lungs.

Segment 7 (1023 nts): Matrix (M1, M2. M42)

The Matrix (M) gene segment is involved in the replication process by interacting with vRNP, regulation of RNA nuclear export, and promoting budding of viral particles to the host cell membrane [1]. The segment encodes two M proteins, the M1 and M2. The M2 acts as an ion channel for H+ uptake [23, 55]. The M1 protein undergoes multimerization and interacts directly with the lipid membrane and surface proteins [55, 56]. These interactions are important to the virion assembly process as it

may facilitate recruitment of crucial viral components. The M1 protein interacts directly with HA and NA by binding to the cytoplasmic tails, which leads to formation of the interior structure of the new virion [57]. Although the M1 protein is responsible for mediating the budding process, the presence of HA and NA is required [55, 57]. The M1 is also required for transportation of vRNPs out of the nucleus by utilizing the NLS [58]. The M2 protein acts as a proton channel which promotes influx of H+ protons, which is required for fusion and the dissociation of vRNPs from M1 to ensure release into the cytoplasm [59, 60]. In addition to M1 and M2, the segment also encodes an alternative protein M42 which acts as an ion channel similar to M2 protein [61]. The full function of M42 is yet to be determined. In swine IAVs, the matrix gene may have a role in increased infectivity in different hosts. One study has shown an Eurasian avian-like H1N1 swine IAV containing the pdm09 H1N1 matrix gene was able to transmit between ferrets and resulted in severe clinical signs [62]. The vast majority of swine H3N2 strains circulating in the United States are known to carry the M gene of pdm09 origin, as a part of their TRIG/pdm backbone [5, 10, 63].

Segment 8 (890 nts): Nonstructural (NS1, NEP/NS2)

Non-Structural (NS) gene segment encodes NS1 and NEP/NS2 proteins. The NS1 protein is an immunosuppressor, which acts by controlling regulation of host gene expression and as an interferon (IFN) antagonist agent [1]. The NS1 is essential for suppressing host innate response by antagonizing type 1 IFN dependent responses, most notably limiting the production of IFN-β [64, 65]. The NS-1 protein limits IFN production by interfering in both pre-transcriptional and post-transcriptional processes [64]. The pre-transcriptional process involves suppressing virus-mediated transcription

factors such as IRF-3 and NFkB pathways [66]. The post-transcriptional process involves inhibition of the host mRNA by directly binding to the poly A tails [67]. In addition, it is involved in a large range of activities including viral replication, modification of viral mRNA and regulating morphology due to its interaction with various factors via the RNA-binding domains, effector domain and the tail domain [64]. The NEP/NS2 is another protein encoded by the gene segment, and is known to interact with vRNPs bound to M1 and transport into the nucleus by interacting with nucleoporins [47]. A study involving an H3N2 swine IAV strain showed that deletions in the NS1 protein decreased the ability of the strain to prevent IFN production by swine cells, reducing its blockage of cellular IFN antiviral activity [68]. Further, viruses containing deletions in NS1 resulted in an attenuated phenotype, leading to the development of a swine influenza vaccine [69].

Replication and Life Cycle of Influenza A

The replication cycle of IAV is a complex process which involves multiple stages starting from attaching to the host cell. As the virion approaches the host cell, the NA cleaves away the polysaccharide residues to allow HA to attach to the SA receptors [7, 70]. Once the HA is successfully attached to the receptor, the M2 channels activate to enable proton influx into the virus, which induces conformational change of the HA. The structural change is triggered by the low pH, which exposes the fusion peptide region, allowing it to merge to the membrane in a collapsing manner to expose the membrane into the cytoplasm [71-73]. Working in concert with M2, the vRNP dissociates from the M1 completing migration to the cytoplasm. The vRNPs are then transported to the host cell nucleus by utilizing the NLS of the viral proteins (mostly NP) via the importin-α

pathway and initiates the transcription and replication process [46, 48, 58, 70]. IAV RNA synthesis takes place in the nucleus, mainly the capped and polyadenylated mRNAs and complementary RNAs (cRNAs) [74]. The capping mechanism of viral mRNAs are unique: they undergo a process of "cap-snatching" which involves PB2 binding to the host mRNAs and PA cleavage to produce short, capped oligonucleotides for the viral polymerase complex to exploit [75, 76]. The RdRp uses the negative sense vRNA as a template for both mRNAs and cRNAs. Once the viral mRNA is modified by capping and polyadenylation, it starts to translate and produce viral proteins while hijacking the host cell machinery. Factors such as NS1 and PA-X promote transcription and translation of viral proteins by down-regulating host protein synthesis [38, 39, 64]. Viral polymerase proteins and NP are imported into the cell nucleus to bind to cRNAs to produce progeny vRNPs. After vRNP assembly, they are exported out of the nucleus and transported via the karyopherin pathway to the membrane via the Rab11 by binding to recycling endosomes [74]. The envelope (M) and surface proteins (HA, NA) are first synthesized in the endoplasmic reticulum of the host cell and then transported to the Golgi apparatus for post-modification [1, 77].

In the late infection cycle, increased expression of HA, NA, and M1 proteins are observed [23]. The virion assembly takes place at the cell membrane in what was thought to be a random manner. However, recent studies have revealed the assembly process might be a more controlled and selective process due to existence of discrete packaging signals [78, 79]. The M1 recruits the transmembrane proteins (HA, NA, and M2) to the virion budding site by direct interaction and by promoting the translocation of vRNPs to the same site [80]. After the assembly of the virus, the budding process occurs at the cell

membrane, initiated by the high concentrations the M1 protein [1]. After the budding process, the HA remains bound to SA receptor and are released into the lumen via the catalytic cleavage of the terminal SA residues by the NA [50]. After release of the virions, the HA0 must be cleaved into HA1 and HA2 subunits to gain proper infectivity, a process mediated by trypsin-like proteases present in the intestinal and respiratory tracts [40, 81-83]. The cleavage site of HA in avian influenza viruses (AIV) determines whether the AIV is a low pathogenic (LPAIV) or highly pathogenic (HPAIV) avian influenza virus. The cleavage site of HA0 in LPAIV is at the monobasic arginine position recognized by trypsin-like proteases [23, 84]. However, the cleavage site in HPAIV contains a string of polybasic amino acids that are recognized and cleaved by a much larger group of enzymes, including furin and subtilisin-like proteases [23], leading to systemic infections with more severe clinical signs.

Influenza in Swine

Swine influenza is a major respiratory disease that is endemic in most parts of the world and causes significant damage to the swine industry [9, 85]. Swine influenza has a high morbidity (almost up to 100%) but mortality is generally low, around 1~4% [63]. Infected pigs show clinical signs similar to those of influenza in humans, displayed as an acute respiratory disease including fever, lethargy and coughing [86]. While the clinical burden of swine influenza happens mostly in pigs that are in the growing stages, breeding sows are also affected leading to decreased reproduction [87]. A systematic study that included 217 published articles showed a seroprevalence of swine IAV was 32.6 to 87.9% in individual pigs and 29.3 to 100% in herds globally [88]. Due to its high morbidity and widespread characteristics, swine influenza can result in significant

economic losses, mostly by affecting weight gain due to loss of appetite and reduced activity. According to the National Pork Producers Council (NPPC), the U.S pork industry lost approximately 5 billion dollars with farms losing 66 percent of its original value during the H1N1 pandemic [89]. The public misconception for food safety during the pandemic led to decreased consumption of swine products, causing an additional damage of one billion dollars [90]. Additionally, swine influenza is a component of the porcine respiratory disease complex and can occur in co-infections with various pathogens such as porcine reproductive and respiratory syndrome (PRRS), porcine circovirus 2 (PCV2), *Mycoplasma hyopneumoniae*, *Bordetella bronchiseptica*, and *Actinobacillus pleuropneumoniae*, which can increase economic damage [91, 92].

Pigs are significant hosts of IAV as they express both types of SA linkages in their respiratory tract, allowing infection with both avian- and human-origin IAV strains and increasing the likelihood of reassortment to occur. For this reason, pigs are thought to be “mixing vessels” for the generation of novel IAVs that can jump between species [93]. However, it is now known that avian and other zoonotic viruses can infect humans without the need for an intermediary host. The susceptibility of pigs to various IAVs has been shown in animal studies, monitoring programs and field cases in swine farms [94-96]. Pigs played an important role for the 2009 pandemic, as the pandemic H1N1 virus (H1N1pdm09) originated from the reassortment of IAVs that were endemic in pigs: a virus known to contain a triple reassortant internal gene (TRIG) constellation that is endemic in North America and a virus of the Eurasian avian-like H1N1 lineage [12]. This reassortant virus seemed to have circulated undetected in swine for many years before jumping into humans [97]. Thus, swine influenza poses a public health risk to humans.

Studies have shown the majority of the risk arises from sites where pigs and human come into direct contact [98, 99]. Particularly, exhibition fairs and live markets poses the biggest risks, as demographic of various origin merges at these sites, including children, elderly and immunosuppressed individuals who are relatively susceptible to swine IAV infections [99]. Sporadic cases in the United States of humans infected with swine IAV, termed variant IAVs, such as H1N1v, H3N2v and H1N2v, have been reported by CDC [100].

Swine influenza can spread very rapidly, with a high R0 around 10.66, and most current control strategies focus on reducing the R0 value below 1 [101]. Vaccination of swine herds is effective in controlling spread of the disease, in addition to animal management strategies such as implementing all-in all-out systems [90, 101]. Vaccination of farm employees may also present an effective control measure to reduce IAV transmission from humans to pigs . Thus, developing and implementing efficient animal management strategies along with developing efficient vaccines for both swine and human would be crucial in preventing swine influenza.

The first cases of swine influenza were reported in 1918 around the same time as the 1918 human pandemic ("Spanish flu) and became endemic worldwide, known as the classical swine H1N1 virus (cH1N1) [92]. This lineage circulated relatively stable for many decades until the introduction of the avian-like H1N1 in Europe in 1979 and the TRIG lineage in North America. Currently, there are 3 major subtypes of swine IAV that are endemic globally and include H1N1, H3N2 and H1N2. Considerable genetic and antigenic diversity exists between and within these subtypes, reflecting the high variability of IAVs [9]. The reason for such diversity may be explained by the

introduction of the TRIG constellation in pigs in the late 90s [102]. The TRIG cassette contains internal genes derived from swine (NP, M, NS), human (PB1), and avian (PA and PB2) influenza viruses forming a constellation of genes that is well conserved. The TRIG cassette is part of the most prevalent backbones in swine IAV strains circulating in the United States. The TRIG constellation can incoorporate surface genes of various origins, a critical factor that may have contributed to increased diversity of swine IAVs in North America [11]. The stability of the TRIG cassette was also proven *in vitro*, when several variants of swine IAVs containing the TRIG cassette successfully adapted and showed robust replication in human respiratory cell lines, while spawning mutations during the process [103]. Once the TRIG cassette was introduced in North American swine, followed by multiple introductions of human-origin IAV into pigs, the new strains reassorted with the endemic swine viruses and led to the establishment of many distinct viral genetic clades [63]. Currently, there are two major H1 lineages circulating in pigs in the U.S., including the 1A (classical H1α, H1β, H1γ, pdm09) and the 1B (delta lineage, H1δ1, H1δ2) and two major H3 lineages, the 1990.4 and 2010 [18, 63, 104]. Of these, pdm09, H1δ1, H1δ2, 1990.4 H3 and 2010 H3 are all resultant from reverse zoonotic transmissions.

In Europe, the cH1N1 was eventually replaced by the avian-origin H1N1 (Eurasian lineage) that emerged in the late 1970s [105]. Currently, different lineages of H1N1 and H3N2 subtypes are endemic in European pigs, including the Eurasian avian-like H1N1, human-origin H1N2/N1, human-origin H3N2, and pdm09 H1N1in different reassortant constellations [63, 85] [3, 106]. In Asia, the cH1N1 reassorted with multiple human and avian lineages in various countries, including the H1N1pdm09, resulting in

the circulation of multiple distinct strains with different genotypes [85]. Also, importation of live pigs from North America and Europe resulted in spread of North American-like and European-like strains in Asia [107, 108]. As with North America and Europe, the introduction of H1N1pdm09 lineage resulted in increased diversity due to reassortment with circulating endemic strains. Information on swine IAV strains circulating within Latin America is scarce. However, a study conducted in 2010 showed that H3N2 and H1N1 subtypes are common in Venezuela, Colombia and Brazil [109]. In Argentina, a unique H3N2 strain was detected and shown to be similar to human H3N2s strains that circulated within the country [110]. The strain was able to transmit and cause severe disease when associated with a bacterial coinfection, being the first case of a wholly human H3N2 virus capable of fully infecting pigs in Argentina. In Brazil a surveillance study identified multiple H1N2 and H3N2 viruses that were circulating undetected after the 2009 pandemic occurred [111]. Thus, as observed in other regions, it is likely diversity of swine IAVs in Latin America is high, whith some lineages resulting from spillover between humans and pigs.

Studying the evolution of swine influenza

Frequent interspecies transmission occurs between humans and pigs. Multiple separate introductions of human IAV to pigs were identified after the 1990s, including seasonal H1 and H3 viruses and the H1N1pdm09 virus [3, 5, 112]. Since its introduction into humans, the H1N1pdm09 virus continues to be transmitted from humans to pigs worldwide, highlighting the close human-swine contact and frequency of spillover of human strains to pigs. These human-to-swine spillover events have resulted in the establishment of many novel virus lineages circulating in pigs globally, contributing to

the great genetic and antigenic diversity of swine IAV [3, 13]. Often the spillover events of human IAV leads to the maintenance and circulation of human-origin IAV gene segments in swine, frequently with mutations acquired [9, 18, 88], suggesting that these molecular changes are necessary for IAV to effectively replicate and transmit in the new species. The ideal constellation of genes may be an important determinant to enable human-to-swine spillover events. As previously mentioned, the TRIG cassette can incorporate HA and NA segments from various strains without significantly altering the fitness of the virus, and this permissibility may be associated with the increase in frequency for the establishment of human-origin lineages in pigs [5, 13, 14, 18]. Similarly, one study has shown that reassortant strains with the HA or NA of human origin but incorporating the H1N1pdm09 backbone of a swine H3N1 virus efficiently replicated and caused disease in pigs, resulting in significantly higher level of nasal shedding compared to the whole human virus [13]. Thus, these results show human-origin genes have the potential to fully adapt and persist in pigs, particularly if paired with swine adapted internal genes. Despite the overwhelming evidence of human-to-swine spillover events and wide circulation of viruses containing human-origin genes in pigs, the molecular determinants of how human origin IAVs can transmit and adapt to pigs are not well understood.

Several studies showed that key amino acid substitutions in the antigenic sites of the HA affect antigenicity [113, 114]. Positions 145, 155, 156, 158, 159 and 189 were shown to affect antigenic phenotypes in human seasonal and swine H3N2 IAVs and were located near the RBS [115, 116]. Among these sites, the 145 amino acid position was shown to be of particular importance as an antibody recognition determinant for swine

H3N2 viruses. Although single mutations were shown to only moderately affect antigenicity, contrarily to what was observed for human H3N2 viruses, mutations at the 145 amino acid position in combination with others (e.g., 159 or 189 positions) were shown to significantly affect antigenic distance in swine H3N2 IAV [117]. Based on these results, currently circulating swine H3N2 strains in the North America can be divided into several antigenic clusters based on their antigenic motifs defined as amino acid positions 145,155,156,158,159 and 189, termed red, cyan, and green clusters. Only two amino acids differences resulted in significant antigenic difference among viruses from the cyan and red clusters and only 3 amino acid differences within the antigenic motif was enough to result in significant antigenic difference between viruses from the cyan and green clusters [116]. Furthermore, the antigenic motifs associated with the red, cyan, and green clusters only occur in 14% of all motifs identified in 271 swine IAV H3N2 strains that circulated between 2009 and 2015 in the U.S. [117], the remaining representing antigenic outlyers, which likely result in even greater antigenic diversity of the H3N2 strains. Similar antigenic characterization was observed in studies conducted on H1 swine viruses, most notably strains from the delta clades were shown to be antigenically distinct from each other despite showing similarities in terms of genotypes [118]. For these viruses, changes in the amino acid positions 131, 140, 141, 273 and 309 were associated with antigenic differences [18]. Thus, predicting and updating vaccine strains based just on the phylogeny is not iedal as it would increase the chance of the vaccine mismatch. Consequently, establishing a coherent system involving these types of antigenic analysis for swine IAV vaccine production is greatly needed to counter the threat of the ever-increasing diversity of swine IAVs. Lack of correlation between

antigenicity and genotype is also frequently observed in other regions of the world, with significant antigenic differences existing within viruses isolated from different countries but within the same lineages, which was the case for H1 viruses isolated from Japan and Hongkong [3].

As demonstrated, the HA is under constant antigenic change, particularly in antibody recognition epitopes that are located on the HA head, close to the RBS. Hence, changes in the antigenic sites of the HA may also affect IAV host range. In H5N1 viruses, for example, changes in residues 225 and 228 are known to switch SA specificity [119]. In another study, a variant H9N2 virus containing 226L mutation replicated 100-fold more compared to the 226 Q variant in differentiated human tracheal cells [42]. Further, mutations at amino acid positions 18, 160, 183 and 262 increased virulence of a seasonal human H3N2 strain in mice [120].

The presence of transmission bottlenecks can also shape the overall genetic diversity of IAVs [98, 121]. Transmission bottlenecks can be defined as constraints that reduce the virus population diversity that is transmitted to another host [122]. These bottlenecks can shape the evolution of the virus as a small sub-population is transmitted from the large diversity of variants available from the initial virus populations [123, 124]. Studies that estimate the transmission bottlenecks for influenza viruses in humans have shown divergent results, with bottleneck sizes varying from 1 to 200 distinct genomes [123, 125] In pigs and horses, transmission bottlenecks were shown to be loose, with many mutations shared among different animals in a transmission chain [124, 126]. Serial transmission of the Eurasian avian-like virus resulted in both transient and fixed mutations, showing allele fixation can rapidly occur in pigs [126].

The numerous human-to-swine spillover events within the United States. coupled with continuing antigenic evolution of these viruses in pigs may facilitate their reintroduction in the human population, posing a threat to public health. Human-origin North American swine H3N2 IAV were recently shown to be antigenically different from current human seasonal and vaccine strains, representing a lack of population immunity and likely low efficacy of human vaccines against these strains [127]. Interestingly, the 1990 swine H3N2 lineages (1990.1, 1990.2 and 1990.4) were shown to be antigenically similar to the human seasonal H3N2 vaccines developed within that era. However, after decades of circulation in pigs, these strains showed significant antigenic difference from current human vaccines mostly due to accumulation of mutations on their antigenic motifs. Importantly, both swine IAV H3N2 lineages that circulate in the United States. (1990.4 and 2010) showed significant fold reduction compared to the closest pandemic preparedness candidate vaccine virus (CVV) [127]. All these factors point to the zoonotic risk swine H3N2 IAVs can pose to humans, as differences in these positions may increase the pandemic potential to the human-origin swine strains. These findings emphasize the importance of continuously monitoring and tracking the evolution of swine IAVs.

CHAPTER 3

MATERIAL AND METHODS

Cell lines

Human embryonic kidney 293T cells (HEK293T) and Madin-Darby Canine Kidney (MDCK) cells were used for transfection of plasmids for generation of the viruses by reverse genetics. MDCK cells were also used for viral growth kinetics. HEK293T cells were obtained from ATCC (Catalog # CRL-3216, ATCC Manassas, VA) and MDCK cells were a kind gift from Dr. Robert Webster (St Jude Children's Research Hospital, SJCRH). Both cell lines were cultured at 37°C with 5% CO_2 using Dulbecco`s modified Eagle`s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine and 1% of antibiotic/antimycotics (ab/am; Sigma-Aldrich, St. Louis, MO) for maintenance. Cells were maintained following standard techniques by regularly splitting and passing at 90% confluency. Briefly, cells were washed twice with phosphate buffered saline (PBS) and detached by adding trypsin-EDTA solution containing 1% ab/am (Sigma-Aldrich, St. Louis, MO), followed by incubation at 37°C for 15 min. After incubation, cells were split at a ratio of 1:20 and re-seeded in T75 flasks (Corning, New York, NY) containing 10 ml of supplemented DMEM.

Harvesting and preparation of Primary swine tracheal epithelial cells (pSTECs)

Primary swine tracheal epithelial cells (pSTECs) were used for viral growth kinetic studies and serial passage studies. pSTECs were harvested from trachea samples

of 5 months-old, influenza-negative pigs kindly provided by the University of Georgia, College of Agriculture. The respiratory apparatus was carefully removed by first clamping the proximal and distal end of the trachea, and esophagus and the vena cava with Kelly forceps to prevent contamination before excising the apparatus. The samples were carefully transported to the lab in an ice box, maintaining a low temperature to delay tissue damage. At the lab, the trachea was carefully removed from the respiratory apparatus and was cut into portions of roughly 7~8 cm. The tracheas were put on a petri dish containing DMEM/F12 supplemented with 5% ab/am and connective tissues were removed. After trimming, the trachea was cut on its length to a "C" shape and was put in 50ml tubes containing a digestion solution of DMEM/F12 media (Thermo Fisher , Waltham, MA) containing 1.5mg/ml of pronase (Sigma-Aldrich,St-Louis, MO) and 5% ab/am (Thermo Fisher, Waltham, MA) and incubated at 4°C for 24 h. After digestion, the remnants of the connective tissue was removed and the inner portion of the trachea was gently scraped with a scalpel to release the pSTECs from the epithelial surface. The cells were then transferred to separate 50ml conical tubes and were washed twice with DMEM/F12 media containing 10% FBS (Sigma-Aldrich, St. Louis, MO) and 10% ab/am solution. After washing, the cells were centrifuged at 300 × *g* for 5 min at 4°C and resuspended in 1ml ammonium-chloride-potassium (ACK) lysis buffer (Thermo Fisher , Waltham, MA) to remove red blood cells. After lysis, 9ml of DMEM/F12 with 10% ab/am solution was added and centrifuged at 300 × *g* for 5 min at 4°C. The cells were then resuspended in 1ml DNase (0.5mg/ml) (Sigma-Aldrich,St-Louis, MO) and digested at room temperature (RT) for 10 min. After digestion, the cells were resuspended in 10ml TEC Basic media (Table 3.1) supplemented with 10% FBS and glutaMAX and NaHCO3

(Thermo Fisher Scientific, Waltham, MA). The resuspended cells were placed in a tissue culture dish without collagen and were incubated for 2 h at 37°C and 5% CO_2 to allow fibroblasts to attach. Once the fibroblasts were fully attached, the remaining pSTECs were collected carefully to not disturb the attached cells in a separate tube. After removing the fibroblasts, the pSTECs were counted using a hematocytometer and aliquoted in 1.5 ml tubes (1.0×10^6 cells per tube) and were centrifuged at 300 × *g* at 5 min at 4°C. The pelleted cells were resuspended in Recovery™ Cell Culture Freezing Medium (Thermo Fisher , Waltham, MA) and put in a Mr. Frosty™ freezing container (Thermo Fisher , Waltham, MA) and gradually frozen in the -80 freezer for a minimum of 12 h before being stored in liquid nitrogen.

Culturing Primary swine tracheal epithelial cells (pSTECs) in transwell supports

For culture and differentiation of pSTECs, collagen-coated (Thomas Scientific, Swedesboro, NJ) transwell inserts (Corning, New York, NY) were used to maintain Air-Liquid-Interface (ALI) conditions. Cells were plated in each insert at 1×10^4 cells/well (for 1.12 cm^2 transwell inserts) in TEC plus media (Table 3.1) by adding 500µl of media to the apical well and 1ml to the basolateral portion. The cells were cultured at 37°C and 5% CO_2 with media being changed every 48 h until cells were 100% confluent. During growth, presence of fibroblasts were checked every 24 h as the fibroblasts may hamper the cells from reaching 100% confluence. When an abundance of fibroblasts were observed, TEC plus media supplemented with hydrocortisone (Stem Cell Technologies, Vancouver, Canada) at a concentration of 96mg/ml was added, as hydrocortisone is known to inhibit growth of fibroblasts [128]. Trans-epithelial electrical resistance (TEER) was measured to ensure confluency of the cells using the EVOM meter (World

Precision Instruments, Sarasota, FL) on extra wells to avoid damage to the cells used in experiments. Once the TEER reached ~1000 ohms, the apical media was removed, and the basolateral media replaced with TEC ALI media (Table 3.1). Cells were cultured at ALI for a minimum of 3 weeks at 37°C with 5% CO_2. The basal media of the transwell inserts were replaced every 48 h. Cilia activity was checked every 2-3 days by observing the cilia after adding 50ul of PBS to the apical wells to generate water bubbles to track ciliostasis.

Staining of cilia of differentiated pSTECs

After 3 weeks of ALI culture, the wells were stained to check for cilia activity. Wells were put in 50ml conical tubes containing 20ml of 4% paraformaldehyde (Thermo Fisher, Waltham, MA) and fixed for 15 min at RT. After fixing, the wells were placed into a clean 12 well plate and permeabilized by adding 100µl of 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO)in PBS to the apical well and incubation at RT for 30 min. The wells were washed with PBS three times and blocked by adding 200µl of 10% bovine serum albumin (BSA, MilliporeSigma, St. Louis, MO) overnight at 4°C. The next day, the wells were stained by adding 200µl of monoclonal Anti-acetylated α -tubulin primary antibody (Thomas Scientific, Swedesboro, NJ) with a dilution of 1:800 and incubated for 2 h at RT or overnight at 4°C. After incubation, wells were washed three times with PBS and incubated by adding 200ul of Anti-Mouse Ig2B conjugated HRP secondary antibodies (Invitrogen, Carlsbad, CA) diluted to 1:200 for 20 min at RT. After washing three times with PBS, 3,3´-Diaminobenzidine DAB solution (DAB Substrate Kit, Peroxidase (HRP), with Nickel; Vector laboratories, Burlingame, CA) were prepared with a dilution of 1/100 in PBS as per manufacturer`s instructions and100 µl was added

to the wells and developed for 10 min at RT. Stained wells were observed under the microscope to check for presence of cilia.

Viruses

Viruses used for the studies were either wild type strains or recombinant strains generated via reverse genetics (rg; Table 3.2). The wild type A/Victoria/361/2011 H3N2 strain (A/VIC/11) was kindly provided by Dr. Richard Webby from St. Jude Children`s Research Hospital. This virus was incorporated into the 2012-2013 human influenza vaccine for the Northern hemisphere [129]. The wild type strain A/Swine/Missouri/A01410819/2014 H3N1 (sw/MO/14) was obtained from the United States Department of Agriculture (USDA) Influenza A Virus in Swine Surveillance System via the National Veterinary Service Laboratories Swine IAV repository (19). This virus was one of the first strains of the 2010.1 lineage detected in pigs [13] and was used as a control since it represents a human-origin virus that had already become adapted to swine. The 2010.1 swine IAV lineage closest human seasonal HA ancestor is similar to the A/VIC/11 strain. Hence, A/VIC/11 and sw/MO/14 posses a similar ancestry. The strain A/turkey/Ohio/313053/2004 (ty/OH/04) is a triple reassortant virus that was detected in turkeys but contained all genes of swine-origin (including TRIG backbone), and was shown to replicate efficiently in pigs and to be highly efficient for generation of recombinant viruses via rg [117, 130, 131]. ty/OH/04 was used as the source for TRIG genes and to generate one of the control viruses for the *in vitro* studies. Four reassortant viruses were generated by rg using cloned cDNAs: VIC11rg, VIC11p, VIC11pTRIG, ty/OH/04p. The VIC11rg was designed to carry all the genes of A/VIC/11. The A/VIC/11

and VIC11rg were used for the study to represent complete human viruses lacking a swine-origin backbone, and for comparison between wildtype and rg viruses. The VIC11p strain encodes the matrix (M) gene segment from A/California/4/2009/H1N1 (A/CA/09) and the remaining seven genes from A/VIC/11. The VIC11p was used to test the part of the M gene segment on pathogenesis in swine. The VIC11pTRIG virus carries the HA and NA from A/VIC/11, the M from A/CA/09, and the remaining five genes of ty/OH/04. The VIC11pTRIG was generated to represent the most common backbone in swine H3N2s currently circulating in the United States. This constellation was also acquired by 2010.1 lineage viruses (the same HA lineage as sw/MO/14) in later generations of reassortants and is now one of the predominant internal gene constellations for that lineage [14]. The ty/OH/04p virus encodes the M from A/CA/09 and the remaining seven genes of ty/OH/04. The M gene was replaced to generate a control containing the same backbone as VIC11pTRIG. Four VIC11pTRIG mutant viruses were designed to carry mutations in the HA region, which were revealed during the study: VIC11pTRIG_A138S, VIC11pTRIG_N165K, VIC11pTRIG_N216K, VIC11pTRIG_DM (Double mutant). All viruses used in this study are described in Table 3-2.

Preparation of reverse genetics plasmids

All plasmids used in this study were based in the bidirectional plasmid vector pDP2002 [132] and were kindly provided by Dr. Daniel R. Perez at Universty of Georgia (UGA). Mutants were generated by using the Phusion Site Directed Mutagenesis kit (Thermofisher, Waltham, MA,) with specific mutagenesis primers (Table 3.3). Each primer pair was designed according to manufacturer`s instructions and was used to

introduce mutations into the pDP2002_A/Victoria/2011_HA plasmid, which contained the wild type HA from A/Victoria/361/2011. The primers were designed to carry a mismatch pair at the target site of the desired mutations and were phosphorylated to ensure proper ligation and circularization. PCR cycling conditions for the mutagenesis reactions are as follows: 95°C for 5 min, 25 cycles of (98°C for 30s, 54°C for 30s and 72°C for 10 min), followed by a final elongation step for 10 min. PCR conditions were the same for all the primers. After amplification, electrophoresis was conducted (100 volt for 30 min) with 1% agarose gels. After confirmation of bands, the products were digested using Dpnl at 37°C for 10min before ligation using T4 DNA ligase provided by the mutagenesis kit. After ligation, the plasmids were transformed via One shot TOP10 competent cells (Invitrogen, Carlsbad, CA, . Two µl of plasmid mixture were added to 50 µl of competent cells and incubated at 4°C for 30 min and subsequently at 42°C for 30s (Heat-Shock Method). After transformation, the plasmids were propagated in culture flasks containing 150ml of Luria Broth (Sigma-Aldrich, St. Louis, MO) supplemented with 1% ab/am (Sigma-Aldrich, St. Louis, MO). Flasks were cultured for 24 h at 37°C. After propagation, plasmids were extracted via the Miniprep kit (Qiagen, Germantown, MD) following manufacturer`s recommendation. After collection, sequences were confirmed for plasmids and primers (Table 3.3) by Sanger sequencing (Psomagen Inc, Rockville, MD,).

Reverse genetics and virus rescue

Reverse genetics was performed to generate each virus using an eight-plasmid reverse genetic system based in the bidirectional plasmid vector pDP2002 [133]. The

reverse genetics system used for the study was kindly provided by Dr. Daniel R Perez, UGA. Briefly, a co-culture of Madin-Darby canine kidney cells (MDCK) and human embryonic kidney 293T (HEK 293T) cells was grown in 6-well plates in Opti-MEM (Thermo Fisher Scientific, Waltham, MA) containing 1% ab/am solution (Sigma-Aldrich St. Louis, MO). Once semi-confluent (~90%), plasmids containing the respective segments were mixed in a separate 1.5 ml tube containing Opti-MEM and TransIT LT1 transfection agent (Sigma-Aldrich St. Louis, MO) up to a total volume of 200 ul. The amount of transfection agent was calculated by 2µl of the agent per 1ug of plasmid. After incubating the mixture at room temperature (RT) for 45 min, 800µl of Opti-MEM was added to make 1ml solution. Media was discarded from the co-culture plate and the 1ml transfection solution was added and incubated at 37 °C, 5% CO_2 for at least 8 h. After the initial incubation, the transfection solution was removed and 2ml of Opti-MEM containing 1% ab/am solution and tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (1µl/ml) was added, and the plate incubated for 72 h. TPCK-trypsin (1µl/ml) was added to the plate every 48 h. Cytopathic effect (CPE) was checked daily. After 72 h, provided apparent CPE was observed, the mixture was collected and tested by hemagglutination (HA) assay. HA assay was performed in 96-well plates in serial 2-fold dilutions in PBS in 50µl volumes. The same volume of 0.5% turkey red blood cells (RBC) was added to each dilution and the plate was incubated at RT for 30 min following standard procedures [134].

Animal Study

The animal study for this dissertation was conducted by the USDA-ARS. A total of eighty 3-week-old, cross-bred pigs, including both sexes, obtained from a herd free of IAV and porcine reproductive and respiratory syndrome virus (PRRSV) were used. Prior to the start of the study, pigs were treated with ceftiofur crystalline free acid (Zoetis Animal Health, Parsippany, NJ) and enrofloxacin (Elanco Animal Health, Greenfield, IN) to reduce bacterial contaminants. Animals were demonstrated to be free of other respiratory pathogens by testing bronchoalveolar lavage fluid (BALF) at the end of the study for PRRSV, porcine circovirus type 2 (PCV2), and *M. hyopneumoniae* nucleic acid by real-time RT-PCR (VetMax, Life Technologies, Carlsbad, CA) and shown to be seronegative to IAV antibodies by a commercial ELISA kit (AI MultiS-Screen kit, IDEXX, Westbrook, ME). Pigs were divided into six groups (A/VIC/11, VIC11rg, VIC11p, VIC11pTRIG, sw/MO/14 and negative control) and housed in biosafety level 2 (BSL2) containment and cared for in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center-USDA-ARS.

Pigs (n=10/group) were inoculated intranasally (1ml) and intratracheally (2ml) with a dose 10^5 50% tissue culture infective dose ($TCID_{50}$)/ml of each assigned virus. Intratracheal inoculation was used to guarantee delivery of virus to the lungs to compare viral titers and pathology in the lower respiratory tract as well as the upper respiratory tract. Five pigs were assigned as negative controls (NC). Inoculation was performed under anesthesia, using an intramuscular injection of a cocktail of ketamine (8 mg/kg of body weight), xylazine (4 mg/kg), and Telazol (6 mg/kg) (Zoetis Animal Health, Parsippany, NJ). At 2 days post inoculation (dpi), five contact pigs/group were placed in separate raised decks in the same room (indirect contacts) with inoculated pigs to

evaluate respiratory transmission. Pigs were checked daily for clinical signs of respiratory disease. Nasal swabs (FLOQSwabs, Copan Diagnostics, Murrieta, CA) were collected daily from 0 to 5 dpi for directly inoculated pigs and daily from 0 to 5, 7, and 9 days post contact (dpc) for respiratory contact pigs, placed in 2 ml minimal essential medium (MEM), and frozen at −80°C until used. Directly inoculated pigs were humanely euthanized at 5 dpi with a lethal dose of pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) and necropsied, and postmortem samples including BALF, trachea and right cardiac or affected lung lobe samples were obtained. Respiratory contact pigs were euthanized at 15 dpc, when serum samples were collected.

Pathological examination of lungs

At necropsy, lungs were removed and evaluated for the percentage of the lung affected with purple-red consolidation typical of IAV infection. The percentage of the surface affected by pneumonia was visually estimated for each lung lobe, and a total percentage for the entire lung was calculated based on weighted proportions of each lobe to the total lung volume [135]. Tissue samples from trachea and lung were fixed in 10% buffered formalin for histopathologic examination. Tissues were routinely processed and stained with hematoxylin and eosin. Microscopic lesions were evaluated by a veterinary pathologist blinded to treatment groups and scored according to previously described parameters [136]. Briefly, lung pathological scores were assigned to 4 different parameters: bronchial and bronchiolar epithelial necrosis or proliferation, suppurative bronchitis or bronchiolitis, peribronchiolar lymphocytic cuffing, and alveolar septal thickening with inflammatory cells (interstitial pneumonia). Individual scores were

summed and a composite score for each pig was computed for lung and trachea microscopic lesions. Trachea and lung tissues were assessed for IAV-specific antigen using immunohistochemistry (IHC) using anti-IAV nucleoprotein monoclonal antibody (MAb) (clone HB-65; ATCC, Manassas, VA,) and scored as previously described [136].

Serology

Serum samples were collected from indirect contact pigs at 15 dpc to check for seroconversion by using hemagglutination inhibition (HI) assay. Prior to HI, sera were treated with receptor-destroying enzyme (Sigma-Aldrich, St. Louis, MO), heat inactivated at 56°C, and adsorbed with 50% turkey red blood cells (RBC). HI assays were performed with either A/VIC/11 or sw/MO/14 as antigens and 0.5% turkey RBCs using standard techniques [137]. Reciprocal titers were divided by 10 and log2 transformed and reported as the geometric mean.

***In vitro* serial passage of viruses**

Viruses VIC11pTRIG and ty/OH/04p were serially passaged in pSTECs to evaluate the evolution of the HA and NA gene segments. Prior to passage, pSTECs were grown and differentiated for 3 weeks in ALI conditions as described above. For the first passage, VIC11pTRIG was seeded with a multiplicity of infection (MOI) of 0.1 and ty/OH/04p was seeded with a MOI of 0.01. MOI was calculated based on the cell count number acquired by trypsinizing the monolayer of pSTECs before proceeding to ALI. Briefly, pSTECs were grown as described above until TEER reached ~1000 Ohms and trypsin-EDTA was added and incubated at 37°C for 30 min to ensure detachment. After incubation, 4 ml of DMEM with 10% FBS was added to inactivate the trypsin and cells

were centrifuged at 500 × *g* for 7 min at RT. Cells were resuspended in 1ml of DMEM/F-12 and counted. The average count of the trypsinized monolayer was 3.5 X 10^5 cells.

For serial passages, viruses were diluted in TEC infection media (Table 3.1) according to MOI and 200µl was added to the apical portion of each 12-well plate transwell insert in triplicates, following with incubation at 37°C 5% CO_2 for 1 h. After incubation, the remaining media was removed, and inserts were washed gently once with PBS to avoid damage to the differentiated layer. After washing, the plates were incubated for 72 h at 37°C and 5% CO_2, and CPE was checked daily. After 72 h post inoculation (hpi), viruses were harvested by adding 300µl of infection media to the apical well and gently pipetted several times to ensure full collection of viruses. The plates were incubated for 30 min at 37°C, 5% CO_2 before collection. A 12-well pSTEC plate was used to conduct up to 3 passages for a single virus since the viruses were passaged in triplicates, a total of nine wells were used for three passages. One well was left for checking TEER and one well left for staining. After the first passage, dilutions of 1/10 were used for subsequent passages of VIC11pTRIG until passage 5, when dilutions were changed to 1/100 after virus titers showed an increase. Dilutions of 1/100 were used for all passages for the ty/OH/04p strain. The viral titers were checked for every passage and dilutions were altered accordingly.

Virus Isolation

Nasal swab samples were filtered with 0.45µm filters and subjected to virus isolation as previously described (26). Briefly, 20 µl of filtered samples were plated in 24-well plates onto confluent MDCK cells washed twice with PBS and incubated for 1 h

at 37°C with 5% CO_2. After incubation, 200µl of Opti-MEM with ab/am and TPCK-trypsin was added to each well. At 48 h of incubation, plates were evaluated for CPE, supernatant removed, and fixed with 4% phosphate-buffered formalin diluted in 0.05% PBS Tween for 30 min at RT. After fixation, plates were washed three times with 0.05% PBS Tween and stained using immunocytochemistry (ICC) as previously described [138]. Plates were incubated with an anti-IAV nucleoprotein MAb (clone HB-65; ATCC, Manassas, VA) at 1:500 dilution in 0.05% PBS Tween containing 1% BSA (diluting solution) at RT for 30 min. After washing with 0.05% PBS Tween three times, plates were incubated with secondary antibody rabbit anti-mouse IgG conjugated horseradish peroxidase (HRP; SouthernBiotech, Birmingham, AL) at 1:250 dilution in diluting solution. The reaction was revealed using 3-amino-9-ethylcarbazole (AEC) substrate (Sigma-Aldrich, St. Louis, MO).

Virus Titration

Titration via $TCID_{50}$ method in tissue culture

Nasal swab, BALF, and tissue culture supernatant from serial passages were titrated by $TCID_{50}$. MDCK cells were seeded with a density of 1.5×10^4 per well in 96 well plates with Opti-MEM with ab/am and incubated for 24 h or until desired confluency (~90%). In a separate 96 well plate, 216µl of Opti-MEM containing TPCK trypsin (1µg/ml) and 1% ab/am was added to each well. After addition of media, 24µl of viruses were added to the first 4 wells of their respective rows. Viruses were 10 fold diluted up to the last well in the row. After dilution, media from the MDCK plates was discarded and 200µl of the diluted viruses were added to their respective wells, starting

from the highest dilution. The plates were incubated for 72 h at 37°C and 5% CO_2. After incubation, plates were read by either ICC as described above or by hemagglutination (HA) assay. For HA assays, 50µl of virus was collected and tested as described above. Titers were calculated via the Reed–Muench method [139].

Titration via quantitative real time PCR (qRT-PCR).

Tissue culture supernatants were subjected to titration via qRT-PCR. 50µl of RNA samples were extracted via the MagMAX™-96 AI/ND Viral RNA Isolation Kit (ThermoFisher, Waltham, MA) following the manufacturer's instructions. The RNA was extracted and eluted via the MagMAX™ Express-96 Deep Well Magnetic Particle Processor (Thermo Fisher, Waltham, MA,). For qRT-PCR, a master mix was prepared by adding 10µl of ToughMix PCR Master Mix (Quantabio, Beverly, MA), 0.5µl each of forward and reverse primers (Table 3.3), 1µl of probe and adding Hyclone™ Nuclease free water (VWR International Inc, Grafton, MA) up to 15µl per sample. After adding 15µl master mix to the qRT-PCR plate (MicroAmp™ Optical 96-Well Reaction Plate, Thermo Fisher , Waltham, MA), 5ul of RNA was added to their respective wells, including standards. Standards were prepared by serial diluting the extracted RNA stock of A/VIC/11 strain (with a known starting titer of 1.08×10^8 $TCID_{50}$/ml) by tenfold, creating dilutions ranging from 10^2-10^7. Plates were sealed and centrifuged at 500 × *g* for 2 min. PCR conditions were as follows: 61°C for 30s, 95°C for 30s, then 45 cycles of 95°C for 10s, 60°C for 20s, and 72°C for 10s, followed by final cooling stage of 4°C for 10s using the Quantastudio3 cycler (Thermo Fisher, Waltham, MA).

NGS and Variant analysis

Virus RNA samples were extracted from tissue culture supernatants either via the MagNA Pure LC RNA Isolation Kit (Roche, Indianapolis, IN) or MagMAX™ AI/ND Viral RNA Isolation Kit t (Thermo Fisher, Waltham, MA) according to manufacturer`s instructions. Multi-segment, one-step RT-PCR (MS-RT-PCR) was conducted for the amplification of the whole IAV genome with the Superscript III High-Fidelity RT-PCR Kit (Thermo Fisher Scientific, Waltham, MA), as previously described [97]. PCR conditions were: 55°C for 2 min, 94°C for 2 min, followed by 35 cycles at 94°C for 30 sec, 50°C for 30 sec, 68°C for 3 min, and final elongation of 4 min at 68°C. Libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, Inc., San Diego, CA) and incubated in the thermo cycler at 98°C for 30s, followed by 8 cycles of 97°C for 10s, 65°C for 30s, 72°C for 30s. The reaction was set up using 40% of the suggested final volume. Libraries were purified and size-selected using 0.7× Agencourt AMPure XP Magnetic Beads (Beckman Coulter Life Sciences, Indianapolis, IN) following manufacturer's recommendation. Collected samples were normalized to 4 nM and pooled after fragment size distribution was analyzed using the High Sensitivity DNA Kit (Agilent, Santa Clara, CA). Pooled libraries were loaded at 15 nM and sequenced using the 300-cycle MiSeq Reagent Kit v2 (Illumina, San Diego, CA) in a paired-end 150-nucleotide run format (150 × 2).

Full genome assembly was performed using a pipeline from previously published methods [97]. Briefly, adapters were removed by Cutadapt [140] and reads were mapped against reference sequences in the NCBI's Influenza Virus Resource at GenBank and de novo assembly was performed using Trinity [141]. Final consensus sequences were generated using Burrows–Wheeler alignment tool (BWA) [142] to map reads to contigs.

Custom scripts were used to track the most abundant nucleotide at each position. Variant calling was performed using LoFreq 2.1.3.1 [143] with a frequency threshold of 0.02 based on replicate sequence runs for the same control samples, a minimum depth of coverage of 100 and a central base quality score of Q30 or higher. Quantification of viral diversity within and between hosts were approached using the consensus sequences and single nucleotide variants within all hosts derived from the variant call data utilizing the DNASTAR Lasergene® bioinformatics package Version 17 (DNASTAR, Madison, WI).

Visualizing and mapping HA protein structures

Three-dimensional (3D) analysis was conducted on the HA protein of the VIC11pTRIG variants identified in the study. DNASTAR Lasergene® Version 17 (DNASTAR, Madison, WI) was used to generate fasta files for HA nucleotide sequences of the VIC11pTRIG variants. DNASTAR Lasergene® Version 17 (DNASTAR, Madison, WI,) and ExPASy (SIB bioinformatics resources, [144] were used to identify and cross-check open reading frames (ORF) in the sequences. The generated protein sequences were submitted to the I-TASSER (University of Michigan, Ann Arbor, M) and visualized with CHIMERA 1.141 (University of California, San Francisco, CA). Respective *.pdb files were generated via the I-TASSER program and were submitted to CHIMERA and files were modified to highlight critical structures and exported as *.py files to create figures. Only protein structures with a positive C-score (Confidence score) were selected for analysis.

Virus growth kinetics

Growth kinetics were conducted to evaluate virus replication in pSTECs and MDCK cells for mutant viruses: VIC11pTRIG_A138S, VIC11pTRIG_N165K, VIC11pTRIG_N216K, VIC11pTRIG_DM. In each experiment, VIC11pTRIG and ty/OH/04p were used as controls. All viruses contained the same internal gene backbone, the TRIG/pdm constellation. The pSTECs were differentiated for 3 weeks in ALI conditions and MOI 0.05 was calculated as described above. Viruses were seeded in pSTECs in triplicates with TEC infection media as described above. For MDCK cells, monolayers were grown in 12 well plates for 24-48 h in Opti-MEM supplemented with ab/am. After reaching desired confluency (~90%), two wells were trypsinized with trypsin-EDTA for 10 min after washing twice with PBS and DMEM with 10% FBS was added to stop reaction. After centrifugation at 500 × *g* for 7 min, MDCK cells were resuspended, and cells were counted. Viruses were diluted to an MOI of 0.05 in Opti-MEM infection media containing TPCK-trypsin (1μg/ml). Diluted virus (500μl) was added to triplicate wells and incubated at 37°C and 5% CO_2 for 1 h and were washed once to remove non-attached virus. Sampling was done at time points 0, 12, 24, 48, and 72 hpi either as described above for pSTECs or by collecting 500μl tissue culture supernatants for MDCK cells. Culture samples for each time point were stored at -80°C until used for titration via qRT-PCR method.

Comparison of HA mutations to sequences of global swine IAV isolates

The sequences from the HA of swine H3 IAV were downloaded from the Influenza Research Database (IRD) [145] on March 30, 2022 to evaluate amino acid residues found at the positions in which mutations were detected in the *in vitro* serial passage study. HA protein sequences were obtained from 2,928 viruses from North

America and 353 viruses from Asia, Europe, South America and Oceania. HA protein sequences were aligned using MegAlign within the DNASTAR Lasergene® package Version 17 (DNASTAR, Madison, WI). Aligned sequences were analyzed using WebLogo version 2.8.2 [146] after adjusting HA for H3 numbering [43].

Statistical analysis

All statistical analyses were conducted using the GraphPad Prism version 9.3.1 (GraphPad, San Diego, CA), with a P-value ≤ 0.05 considered significant. Statistical methods used to analyze log10 transformed virus titers, lesion scores, log2 transformed HI reciprocal titers, etc, include nonparametric one-way analysis of variance (ANOVA), two-way ANOVA and nonparametric t-tests. Results shown to be significant were subjected to pair-wise comparisons using the Tukey–Kramer test or the Dunn's test with Bonferroni correction.

Table 3.1 List of cell culture media and components used for the study

Media	**Component**
DMEM Complete	DMEM, 1% Antibiotic/Antimycotic, 1% L-glutamine, 5% FBS
Opti-MEM infection	Opti-MEM,1% Antibiotic/Antimycotic, TPCK[3]
Opti-MEM Growth	Opti-MEM,1% Antibiotic/Antimycotic
TEC Basic	DMEM/F-12, 200mM glutaMAX, 7.5% $HaHCO_3$, 1% Antibiotic/Antimycotic
TEC Plus	DMEM/F-12, 200mM glutaMAX, 7.5% $HaHCO_3$, 1% Antibiotic/Antimycotic, Insulin[1] (1pug/ml), Transferrin[2] (5ug/ml), Cholera toxin[3] (100 ng/ml), Epidermal growth factor[4] (25ng/ml), Bovine pituitary extract[5] (15mg/500ml), Retinoic acid[6] (5×10^{-7}M)
TEC ALI	DMEM/F-12, 200nM glutaMAX, 7.5% $HaHCO_3$, 1% Antibiotic/Antimycotic, 2% Nu Serum, Retinoic acid (5 × 10-7M)
TEC Infectious	DMEM/F12, 1% Antibiotic/Antimycotic, 0.025% BSA[7]

[1,2,3,6,7] Sigma-Aldrich; [4] Thermofisher; [5] Corning

Table 3.2 List of viruses used for the study.

Viruses	Gene Constellation								Chapter
	PB2	PB1	PA	HA	NP	NA	M	NS	
A/VIC/11[a]	A/VIC/11	A/VIC/11	A/VIC/11	A/VIC/11	A/VIC/11	A/VIC/11	A/VIC/11	A/VIC/11	4
VIC11rg[a]	A/VIC/11	A/VIC/11	A/VIC/11	A/VIC/11	A/VIC/11	A/VIC/11	A/VIC/11	A/VIC/11	4
VIC11p[b]	A/VIC/11	A/VIC/11	A/VIC/11	A/VIC/11	A/VIC/11	A/VIC/11	A/CA/09	A/VIC/11	4
VIC11pTRIG[c]	ty/OH/04	ty/OH/04	ty/OH/04	A/VIC/11	ty/OH/04	A/VIC/11	A/CA/09	ty/OH/04	4, 5
VIC11pTRIG_A138K*	ty/OH/04	ty/OH/04	ty/OH/04	A/VIC/11	ty/OH/04	A/VIC/11	A/CA/09	ty/OH/04	4
VIC11pTRIG_N165K*	ty/OH/04	ty/OH/04	ty/OH/04	A/VIC/11*	ty/OH/04	A/VIC/11	A/CA/09	ty/OH/04	5
VIC11pTRIG_N216K*	ty/OH/04	ty/OH/04	ty/OH/04	A/VIC/11*	ty/OH/04	A/VIC/11	A/CA/09	ty/OH/04	5
VIC11pTRIG_DM*[c]	ty/OH/04	ty/OH/04	ty/OH/04	A/VIC/11*	ty/OH/04	A/VIC/11	A/CA/09	ty/OH/04	5
sw/MO/14	A/CA/09	A/CA/09	A/CA/09	sw/MO/14	A/CA/09	sw/MO/14	A/CA/09	A/CA/09	4
ty/OH/04p	ty/OH/04	ty/OH/04	ty/OH/04	ty/OH/04	ty/OH/04	ty/OH/04	CA/09	ty/OH/04	4,5

A/VIC/11, A/Victoria/361/2011 H3N2; ty/OH/04, A/Turkey/Ohio/313053/2004 H3N2; A/CA/09, A/California/04/2009 H1N1; A/VIC/11 *HA containing the A138S, N165K and/or N216K mutations.

[a] Viruses carrying internal genes and surface genes of A/Victoria/361/2011 H3N2; [b] Virus carrying internal genes and surface genes of A/Victoria/361/2011 H3N2 except for the matrix gene from A/California/04/2009 H1N1; [c] Double mutant carrying the N165K and N216K amino acid mutations.

Table 3.3 List of primers and sequences used for the study.

Name of primer	Type	Sequence	Purpose[1]
Phos_Vic11_A138S_F[1]	Mutagenesis	5'-GGAACAAGTTCTTCTTGCATAAGGA-3'	Introduce A138S mutation
Phos_Vic11_A138S_R[1]	Mutagenesis	5'-GTTTTGAGTGACTCCAGTCCAATTG-3'	Introduce A138S mutation
Phos_Vic11_N165K_F[1]	Mutagenesis	5'-CCCAGCATTGAAAGTGACTATGCCA-3'	Introduce N165K mutation
Phos_Vic11_N165K_R[1]	Mutagenesis	5'-TATTTGAAGTTTAATTGGGTCAACC-3'	Introduce N165K mutation
Phos_Vic11_N216K_F[1]	Mutagenesis	5'-TGTAATCCCGAAAATCGGATATAGA-3'	Introduce N216K mutation
Phos_Vic11_N216K_R[1]	Mutagenesis	5'-GCTTGTTGGCTTCTTTTGGTAGATAC-3'	Introduce N216K mutation
T7_Seq	Sequencing	5-TAATACGACTCACTATAGG-3'	To sequence pDP2002 plasmid
SeqPol1	Sequencing	5'-GAGGTATATCTTTCGCTCCG-3'	To sequence pDP2002 plasmid
M+25_F	Sequencing	5'-AGATGAGTCTTCTAACCGAGGTCG-3'	For quantitative Real-time PCR
M-124 M_Ca/04_CJC	Sequencing	5'TGCAAAGACACTTTCCAGTCTCTG-3'	For quantitative Real-time PCR
M+64	Probe	5'-/56-FAM/TCA GGC CCC CTC AAA GCC GA/36-TAMSp/-3'	For quantitative Real-time PCR
Vic11_HA_Seq_F1	Sequencing	5'-AGCAAAAGCAGGGGATAAT-3'	HA specific primer for A/VIC/11
Vic11_HA_Seq_F2	Sequencing	5'-GAGATCTAATAATAGTTTCTTTA-3'	HA specific primer for A/VIC/11
Vic11_HA_Seq_R3	Sequencing	5'-GGGTGTTTTTAATTAATGCACTC-3'	HA specific primer for A/VIC/11
Adapter STRAND 1	NGS[2]	5'-/5Phos/GATCGGAAGAGCACACGTCT-3'	Adapter extension
Adapter STRAND 2	NGS	5'-ACACTCTTTCCCTACACGACGCTCTTCCGATC-3'	Adapter extension
NGS_F	NGS	5'-AATGATACGGCGACCACCGAGATCTA CACTCTTTCCCTACACGACGCTCTTCCGAT-3'	NGS universal primer

Idx02-Long-HPLC	NGS	5'-CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-3'	Indexing primer
Name of primer	*Type*	*Sequence*	Purpose[1]
Phos_Vic11_A138S_F[1]	Mutagenesis	5'-GGAACAAGTTCTTCTTGCATAAGGA-3'	Introduce A138S mutation
Phos_Vic11_A138S_R[1]	Mutagenesis	5'-GTTTTGAGTGACTCCAGTCCAATTG-3'	Introduce A138S mutation
Phos_Vic11_N165K_F[1]	Mutagenesis	5'-CCCAGCATTGAAAGTGACTATGCCA-3'	Introduce N165K mutation
Phos_Vic11_N165K_R[1]	Mutagenesis	5'-TATTTGAAGTTTAATTGGGTCAACC-3'	Introduce N165K mutation

A/VIC/11, A/Victoria/361/2011 H3N2; [1] Mutations on the HA of A/ VIC/11; [2] Next Generation Sequencin

CHAPTER 4

TRANSMISSION OF HUMAN INFLUENZA A VIRUS IN PIGS SELECTS FOR ADAPTIVE MUTATIONS ON THE HA GENE SEGMENT

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Abstract

Influenza A viruses (IAV) are known to cause respiratory diseases in many host species, including humans and pigs. The spillover of IAV between swine and humans have been a concern for both public health and the swine industry. With the emergence of the triple reassortant internal gene (TRIG) constellation, establishment of human-origin IAVs in pigs has become more common, leading to increased viral diversity. However, little is known about the adaptation processes that are needed for a human-origin IAV to transmit and become established in pigs. We generated a reassortant IAV containing surface gene segments from a human IAV strain and internal gene segments from the 2009 pandemic and TRIG IAV lineages and demonstrated that it can replicate and transmit in pigs. Sequencing and variant calling analysis identified a variant that emerged during replication in pigs, which was mapped to a region near the receptor binding site of the hemagglutinin (HA). The variant was present in all contact pigs and replicated more efficiently in differentiated swine tracheal cells compared to the virus containing the wild type human-origin HA. These results show that variants are selected quickly after replication of human-origin HA in pigs, leading to improved fitness in the swine host, likely contributing to transmission.

Key words: Virus evolution, H3N2, TRIG, Primary swine epithelial cell, viral growth kinetics, reverse genetics, adaptation

Introduction

Influenza A viruses (IAV) are enveloped viruses known to cause respiratory disease in several species, including humans and pigs. IAV has a wide host range and can adapt and cross between species [2]. IAV circulates worldwide in pigs, with various strains and subtypes prevalent among swine populations. The major subtypes of IAV of swine include H1N1, H3N2, and H1N2, and considerable genetic and antigenic diversity exists within these subtypes [9].

Hemagglutinin (HA) is the major surface glycoprotein of IAV and is a critical component in determining host specificity due to its role in initial binding and entry into the host cell [1, 2, 5]. The HA binds via its receptor binding site (RBS) to sialic acids (SA) in the host cell in the conformation of either SA-α-2,3 linkage or SA-α-2,6 linkage. SA-α-2,3 conformation is dominantly expressed in avian species, particularly in the respiratory and intestinal epithelial cells. SA-α-2,6 conformation is dominantly expressed in humans, usually in the upper respiratory tract [6]. Thus, the host-specificity of the HA is affected by its ability to bind to these receptors [7]. Specific amino acid substitutions in or near the RBS can change receptor-binding preference and facilitate host jumps [5]. Critical substitutions have been pointed as important in defining the receptor binding profile and to enable species crossing, such as the 226 amino acid position from H3 and H9 IAVs [8, 42].

Pigs are a unique host for IAV as they are known to express both types of SA linkages, allowing infection with both avian and human origin IAV strains [94-96], increasing the likelihood of reassortment to occur. One example was the generation of the triple-reassortment internal gene constellation (TRIG) that emerged in the late 90s

[102]. The TRIG cassette contains internal gene segments derived from swine (matrix -M, non-structural -NS, and nucleoprotein -NP), human (polymerase basic 1 -PB1), and avian (polymerase acidic -PA and PB2) IAVs forming a gene constellation that has remained well conserved. Previous studies have shown the TRIG cassette is prone to stably incorporate HA and NAs from various origins, potentially explaining the increased evolutionary rate of swine IAV and increased diversity after its introduction [11]. The TRIG cassette has also contributed to the generation of the pandemic H1N1 virus (H1N1pdm09) [12]. Since then, genes of the H1N1pdm09 have been incorporated into the backbone of swine IAVs in North America [10], and most of the swine strains circulating in the United States contain a combination of internal genes of the TRIG and H1N1pdm09 lineages, with most of the genetic diversity arising from mutations in the HA and NA [147].

Frequent interspecies transmission is known to occur between humans and pigs. Numerous introductions of human IAV to pigs were identified from 1990 to 2011, including seasonal H1 and H3 viruses and continual detection of H1N1pdm09 [3, 9, 15]. These human-to-swine spillover events have resulted in the establishment of many novel virus lineages circulating in pigs globally, contributing to the great genetic and antigenic diversity of swine IAV [3, 13]. One of such lineages resulted from a recent spillover from a human H3N2 seasonal virus that became one of the predominant H3N2 lineages in US swine and continues to evolve since its first detection in 2012 [13, 14]. Although IAV are frequently exchanged between humans and pigs, most human-origin viruses reassort with swine strains after the spillover and the persisting human-origin genes show significant changes compared to the human IAV ancestors, particularly the surface genes. Yet, the

molecular determinants enabling the adaptation of human-origin IAVs to pigs are still not clear.

Our previous work has shown that wholly human H3N2 viruses do not typically replicate efficiently in pigs, and transmission is rarely observed [13]. Thus, to evaluate the molecular changes during replication and transmission of human-origin H3N2 surface genes in pigs, a reassortant strain was generated via reverse genetics containing human seasonal HA and NA genes in a common internal genes' constellation found in U.S. swine currently. We showed that this reassortant virus resulted in transmission in pigs and identified mutations in the HA gene that seem to improve transmission and replication in primary swine tracheal epithelial cells (pSTECs). Our results suggest that advantageous mutations are selected quickly in the HA gene of human seasonal IAV during replication in swine.

Material and Methods

Methods used for this Chapter are described in detail in Chapter 3.

Results

The reassortant VIC11pTRIG replicated and caused disease in pigs

Pigs (n=10/virus) were challenged with A/VIC/11, VIC11rg, VIC11p, VIC11pTRIG, and sw/MO/14. BALF was collected from directly inoculated pigs at 5 dpi, when the percentage of lung lesions was assessed (Fig. 4.1A-B). Confirming our previous results [13], viral titers were only detected in the BALF of 2 pigs inoculated with the A/VIC/11 and VIC11rg wholly human seasonal viruses, and from only 1 pig

inoculated with the VIC11p. In contrast, 7 pigs from the VIC11pTRIG-inoculated group had detectable virus titers in the BALF, while titers were detected in all pigs from the sw/MO/14 inoculated group (Fig. 4.1A). Pigs inoculated with the sw/MO/14 showed significantly higher titers compared with all other groups. Even though replication was confirmed in few pigs inoculated with A/VIC/11, VIC11rg, or VIC11p, titers were low (below 10^2.5 $TCID_{50}$/ml), bringing the group average to below the limit of detection (Fig. 4.1A). Similarly, IAV-specific antigen staining was detected by IHC in the lungs of sw/MO/14-challenged pigs, but signals were not observed in any of the other challenged pigs (Table 4.1).

As expected, sw/MO/14 induced high percentage of pneumonia in all pigs, significantly higher compared to all other groups (Fig. 4.1B). The average percentage of macroscopic lesions were very low in the groups challenged with A/VIC/11, VIC11rg, VIC11p, VIC11pTRIG, with no significant difference compared with the negative (non-challenged) group. Similarly, pigs infected with sw/MO/14 showed the highest average microscopic lung and trachea lesion scores (Table 4.1), significantly higher than the other groups. Although some pigs infected with A/VIC/11 and VIC11pTRIG showed slightly higher trachea lesions scores compared to the other groups challenged with viruses containing the A/VIC/11 HA (Table 4.1), no significant differences were observed among these groups nor compared to the negative controls.

The reassortant VIC11pTRIG transmitted between pigs

Nasal swab viral titers were assessed in directly inoculated pigs from 1 to 5 dpi and in respiratory contacts from 1 to 5, 7, and 9 dpc. Like what was observed for

replication in the lungs, only a small number of pigs directly inoculated with the A/VIC/11 or VIC11rg shed low virus titers, with only 3 and 2 pigs, respectively, testing positive at any time-point (Fig. 4.2A). Compared to pigs inoculated with wholly human seasonal virus (A/VIC/11 or VIC11rg), more pigs directly inculated with VIC11p shed virus, with almost all pigs (8 out of 10) being positive at least in one time-point. However, no statistical difference was observed compared to the A/VIC/11 or VIC11rg groups. In contrast, virus was detected in the nasal swabs of all pigs inoculated with VIC11pTRIG and sw/MO/14 at all time-points, and viral titers were significantly higher in comparison with the other three groups for most time-points (Fig. 4.2A). Consistent with little to no shedding observed in A/VIC/11-, VIC11rg-, or VIC11p-directly inoculated pigs, none of the respiratory contact pigs (n=5/group) that were in the same room as these groups were positive at any time-point (Fig. 4.2B). In contrast, all pigs in contact with pigs inoculated with VIC11pTRIG or sw/MO/14 were positive in at least one time-point, starting mostly at 4dpc (Fig. 4.2B). Only one pig in the VIC11pTRIG contact group was positive at 3 dpc (data not shown). Confirming these results, all pigs in these two groups were seropositive by hemagglutination inhibition (HI) assay at 15 dpc (Fig. 4.2C).

Minor variants are selected quickly after replication of the reassortant V*IC11pTRIG in pigs*

NS samples from 5 directly inoculated pigs collected at 1, 3, and 5 dpi, and 5 respiratory contacts collected at 5, 7 and 9 dpc in the VIC11pTRIG group were selected for sequencing by NGS. Samples from directly inoculated pigs with highest $TCID_{50}$/ml

titers in any given time-point (>10^2.75 $TCID_{50}$/ml) were selected for sequencing, and all respiratory contacts. Of the 30 NS samples selected for NGS sequencing, 9 samples from directly inoculated pigs (pigs 375, 378, 340 in all selected time-points) and 12 samples from respiratory contact pigs (pigs 407, 408, 409, 410 in all selected time-points) were amplified by MS-RT-PCR, and 18 produced complete genome assemblies by NGS. Viral genomes were analyzed for HA and NA variants. A total of 3 dominant variants were discovered in the HA: A138S, V186G, and F193Y. A138S emerged in two directly inoculated animals, rose to frequencies of 29% and 77% by 5 dpi, becoming dominant in one animal (Fig. 4.3A). A138S was fixed (99% of viruses) in all 4 contact pigs soon after transmission (Fig. 4.3B). The V186G mutation was present in 2.6% of viruses in one directly inoculated animal at 5 dpi, was not detectable in the contact pigs soon after transmission but became dominant in one animal on 9 dpc with a frequency of 60% (Fig. 4.3C-D). The F193Y mutation was only detected in one directly inoculated pig at a frequency of 56% by 3 dpi, declining on 5 dpi to 27% (Fig. 4.3E). F193Y was transmitted to one pig with a frequency of 9% at 5 dpc and rose to a frequency of 4% in another animal by 9 dpc (Fig. 4.3F). In addition to the major variants, several low-frequency variants were identified along the HA, in a total of 46 positions (Table 4.2). No dominant variants were observed in the NA or other viral gene segment.

We plotted the location of the 3 mutations on the HA head and found all residues were located close to H3 antigenic and binding sites (Fig. 4.4). The A138S mutation is located under the 220 loop which is one of the major structures forming the receptor binding site (RBS). The F193Y and V186G are located next to the 190 loop, another structure that forms the RBS.

Mutation A138S improves replication of the human seasonal HA of VIC11pTRIG in swine cells

Viral growth kinetics were evaluated in pSTECs and MDCKs to compare the replication of the A138S variant (VIC11pTRIG_A138S) in comparison to VIC11pTRIG (Fig. 4.5). ty/OH/04p was used as control and contains the same backbone as the other two viruses. The VIC11pTRIG_A138S showed more efficient replication in pSTECs at all time-points (Fig. 4.5A) in comparison to VIC11pTRIG containing the human seasonal HA, with the highest difference at 72 hpi. Replication of VIC11pTRIG_A138S was like ty/OH/04p up to 24 hpi but ty/OH/04p showed significantly higher titers at 48 and 72 hpi. No significant difference in replication kinetics was observed in MDCK cells for any of the viruses (Fig. 4.5B).

Discussion

Determining the evolutionary processes of influenza virus cross-species transmission is key to understanding the mechanisms that control the emergence of new influenza viruses and establishment in a new host population. Spillover events of IAV between humans and swine are common and contribute to the extensive diversity of IAV circulating in pigs. The TRIG backbone seems to have an important part in expanding this diversity as it has the capability to incorporate HA and NA segments without destabilizing the overall fitness of the strains [5, 9, 13, 14]. While acquiring an efficient and permissive internal gene constellation seems to be a crucial step in the adaptation of IAV to a new species, the molecular mechanisms that drive viral evolution after transmission of human-origin viruses in swine in not well understood. Thus, we

generated a novel reassortant virus (VIC11pTRIG) that contains human seasonal HA and NA in a backbone highly adapted to swine and assessed its evolution during replication and transmission in pigs. Our results show that this ideal backbone significantly increases fitness of human seasonal H3N2 in pigs and is crucial for the transmissibility in pigs, allowing for the further evolution and selection of HA variants.

In this study, we have shown that acquiring the ideal combination of genes is likely the first step for the adaptation of human-origin IAV to successfully replicate and transmit in pigs. The VIC11pTRIG group showed significantly higher viral titers in nasal swabs and BALF compared to groups with full constellation of human-origin internal genes or with the addition of the H1N1pdm09 matrix gene. More importantly, the VIC11pTRIG was the only VIC11 reassortant able to transmit among pigs. The fitness advantage of the TRIG cassette and the importance of an ideal gene constellation had been confirmed previously in co-infection studies, in which only an H3N2 virus with a particular constellation containing the TRIG cassette was able to transmit between pigs [102]. Here, the addition of the H1N1pdm09 matrix gene seems to have increased the replication efficiency of the human-origin internal gene constellation since a higher number of infected pigs in the VIC11p group shed higher viral titers compared to other groups with human seasonal internal genes (A/VIC/11 and VIC11rg), although differences were not statistically significant. However, although VIC11p-directly inoculated pigs shed virus, there was no transmission. Interestingly, although transmission was confirmed in the VIC11pTRIG reassortant group to similar levels as in the swine-adapted sw/MO/14 group, infection resulted in little to no lung lesions and significantly lower titers in the lungs compared to the swine-adapted virus. These

findings are consistent with previous studies that showed that reassortant viruses carrying the A/VIC/11 HA showed replication in the upper respiratory tract and limited replication in the lungs [13].

The HA is a major factor for host specificity [148, 149] and would be a prime target for evolution as a human seasonal virus adapts to the swine host. Specific amino acid substitutions at or near the RBS have been shown to alter receptor binding or antigenicity of influenza viruses [6, 116, 150], which likely affects host specificity. Hence, it is reasonable to infer that once the virus acquires a gene constellation that allows effective replication, in this case the pTRIG backbone, mutations that favor receptor binding in the new host may be the next step to an efficient transmission. Here, the 3 dominant mutations that arose, A138S, V186G, and F193Y, were all located within the antigenic site B of H3N2 [151], near or within the RBS. Thus, it is possible that these mutations, particularly the A138S that was fixed in respiratory contact animals soon after transmission, may have altered the conformation of the HA and its interaction with the host cell. Interestingly, the 138 position was shown previously to affect infectivity [152-154]. The advantageous effect of the A138S mutation for replication in swine was confirmed in growth kinetic assays in pSTECs, a system that mimics the swine respiratory tract, which suggests that this mutation was crucial for the transmissibility of the VIC11pTRIG reassortant virus in our study. The V186G mutation has been detected in surveillance studies as a variant of A/VIC/11-like strains [155], and was associated with replication and evolution in an immunocompromised individual [156]. Although this variant was not transmitted in pigs, it quickly became dominant in a contact animal and it could represent one of many different evolutionary pathways that later could be selected

by the swine environment as an advantageous variant, as has been proposed for transmissibility of avian H1N1 in mammals [157]. The F193Y mutation was dominant in one directly inoculated pig and was potentially transmitted in low frequency. However, it did not become dominant in contact pigs, suggesting this evolutionary pathway did not present an advantage in the swine host.

Although the reassortant virus that we studied here contains human seasonal HA and NA, the rest of the genome was formed by well-adapted swine genes, limiting our ability to evaluate evolution outside of the surface genes. Nevertheless, using an artificial virus with a background known to be effective in pigs allowed us to examine the immediate virus evolution after initial replication in the swine host. As expected, the diversity in the HA was significantly higher compared to other segments, including the NA, and no mutations were fixed in any other segments (data not shown). However, it is unknown whether the A138S HA mutation would have become fixed if a wholly human virus was tested.

Overall, this study suggests that for a human-origin virus to become adapted to pigs it needs an efficient internal gene constellation capable of enhancing replication, allowing for the HA diversification and selection of advantageous variants that will be transmitted between pigs. However, our study is limited to a single transmission event and further studies are needed to accurately confirm these evolutionary processes and the molecular mechanisms required for the adaptation of human-origin viruses in swine.

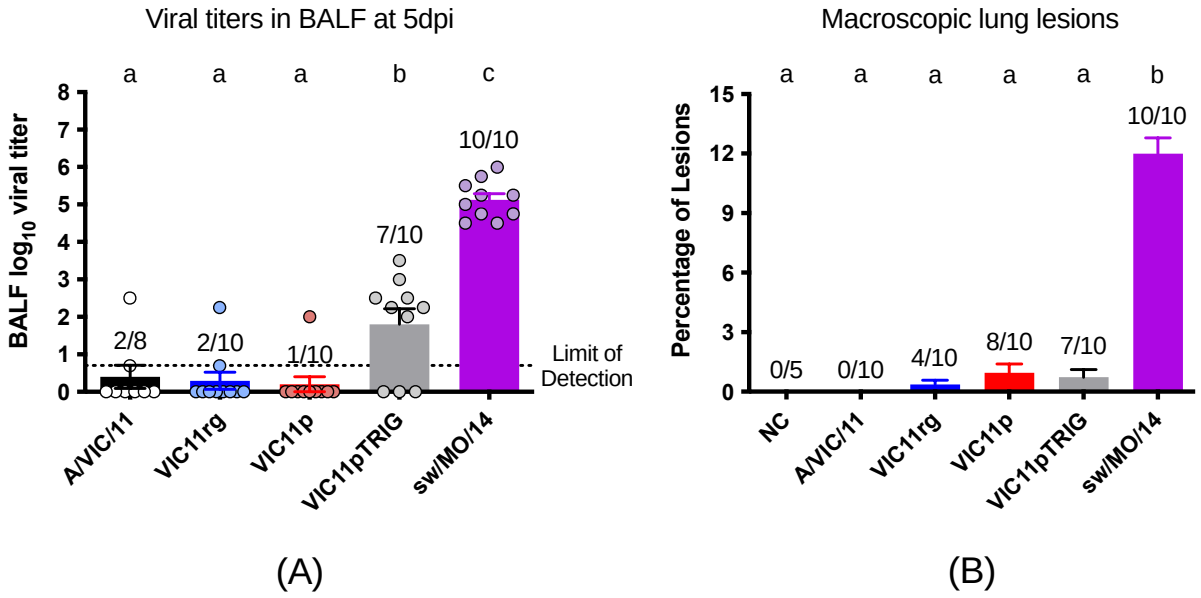

Figure 4.1. Viral titers in the lungs and macroscopic lung lesions of pigs directly inoculated with reassortant viruses. (A) Viral titers in bronchoalveolar lavage fluid (BALF) collected at 5 days post infection (dpi) from pigs directly inoculated with A/VIC/11, VIC11rg, VIC11p, VIC11pTRIG, or sw/MO/14. Values are shown as mean $TCID_{50}$/ml titers ± standard error of the mean, with scattered dots representing individual pigs. (B) Percentage of the lungs affected with purple-red consolidation at 5 dpi. Numbers indicated above the error bar depicts the number of positive or affected pigs/total number of pigs in group. Different lowercase letters indicate significant difference at $P<0.05$.

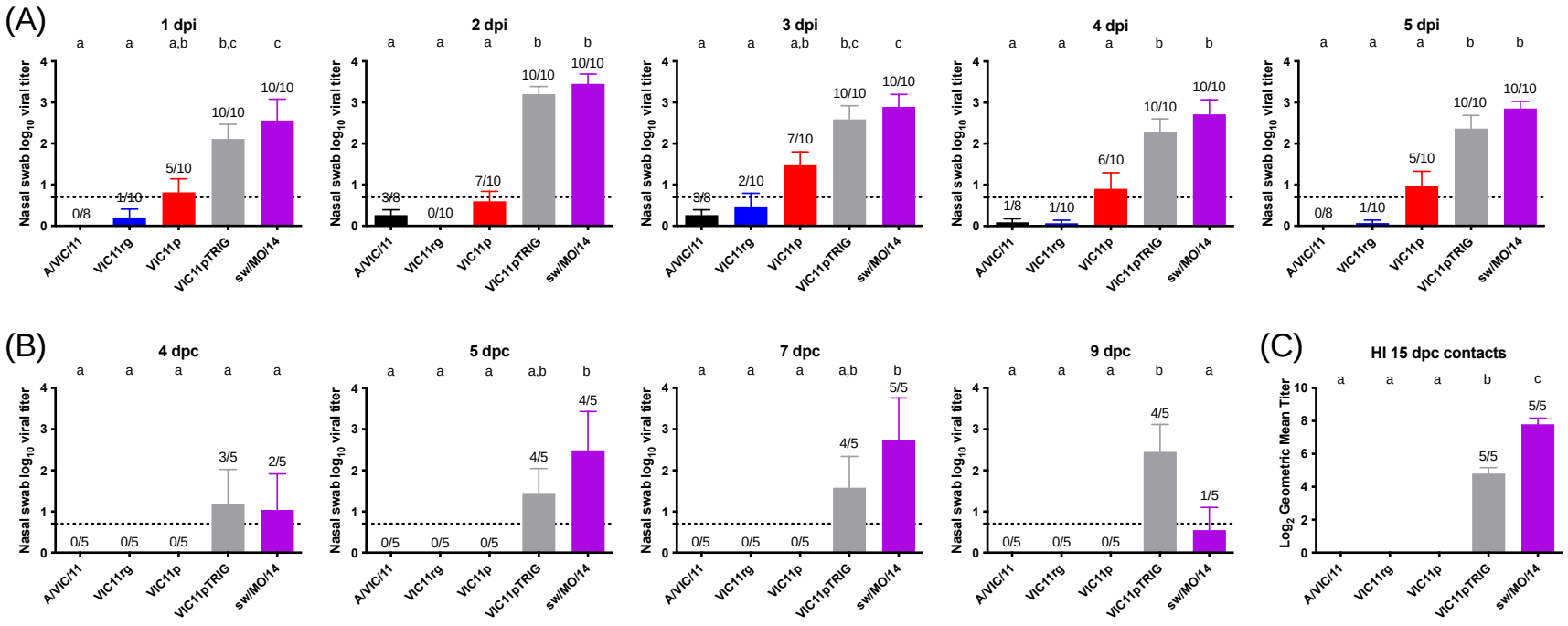

Figure 4.2. Viral titers in nasal swabs of directly inoculated and respiratory contact pigs after infection with human-origin reassortant viruses and seroconversion of respiratory contact pigs. Nasal swabs samples were collected from (A) directly inoculated pigs daily from 1 to 5 days post inoculation (dpi) and from (B) respiratory contact pigs daily from 1 to 5, then 7- and 9-days post contact (dpc). (C) Antibody response in respiratory contact pigs at 15 dpc measured by HI assay. Groups were inoculated with A/VIC/11, VIC11rg, VIC11p, VIC11pTRIG, or sw/MO/14. Values are shown as mean $TCID_{50}$/ml titers or geometric mean titers ± standard error of the mean. Numbers indicated above the error bar depicts the number of positive pigs/total number of pigs in group. Different lowercase letters indicate significant difference at $P<0.05$.

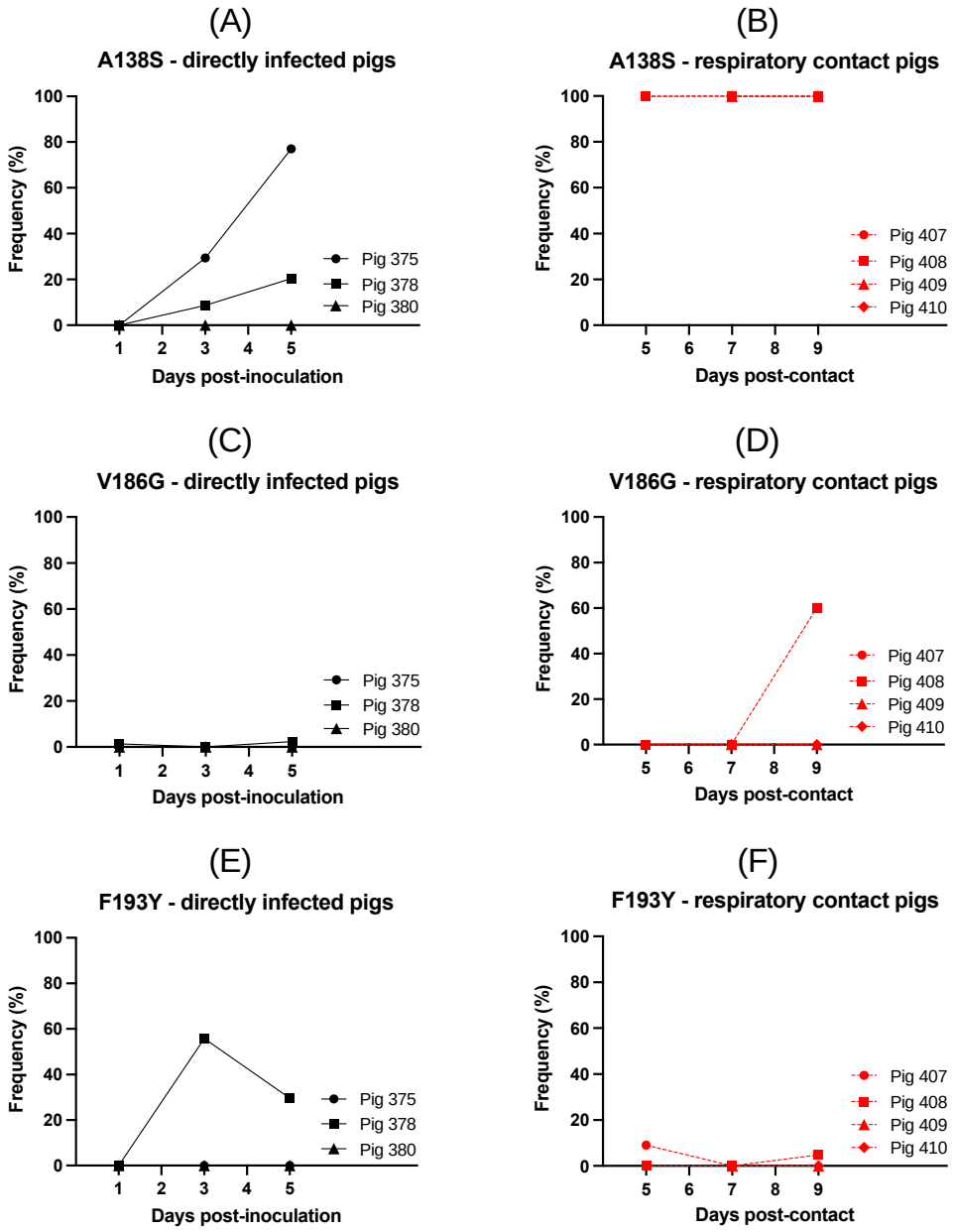

Figure 4.3. Variants identified in directly inoculated and respiratory contact pigs in group infected with the VIC11pTRIG reassortant. Dominant variants detected in each directly inoculated and respiratory contact pigs are shown. Variants with a frequency higher than 50% that were detected in nasal swabs in at least one occasion were considered dominant. (A,B) depicts mutation A138S; (C,D) depicts mutation V186G; (E,F) depicts mutation F193Y. All positions are based on H3 numbering.

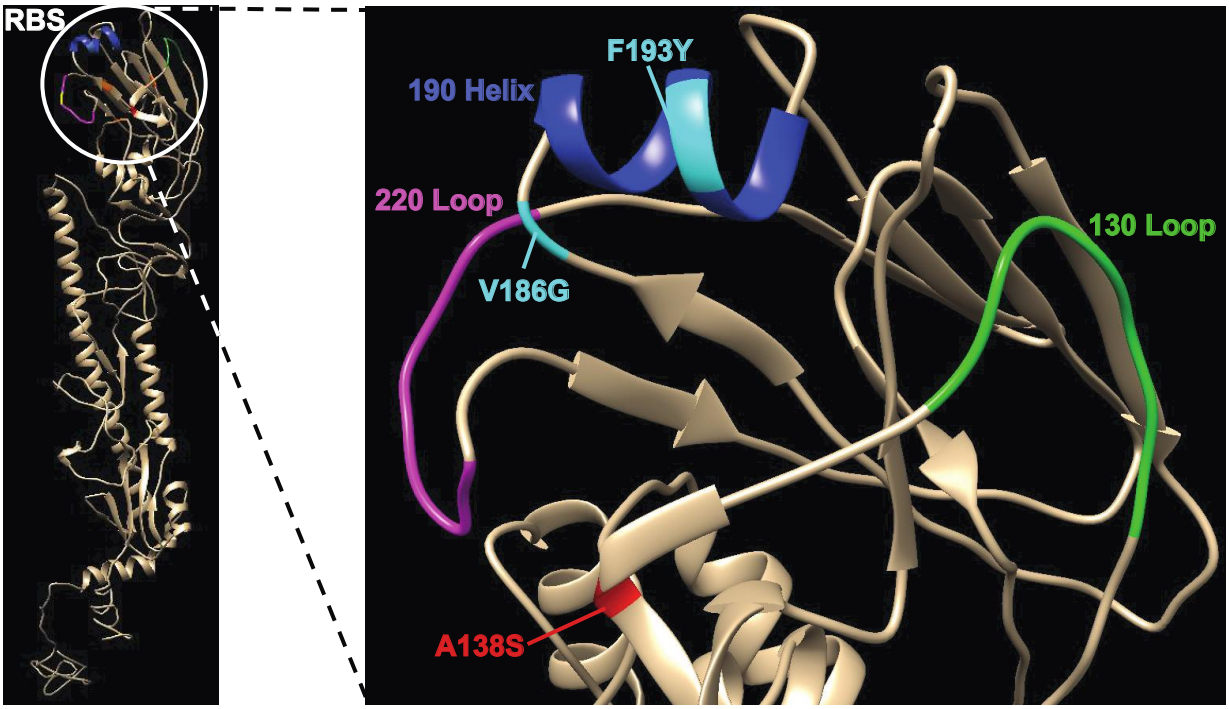

Figure 4.4 Location of mutations identified on the VIC11pTRIG HA. Dominant variants identified in this study on the HA of the Vic11pTRIG are highlighted on the structure of the human A/Victoria/361/2011 H3. Red, residue A138S; cyan, residues V186G and F193Y. The 190 helix, 130 loop, and 220 loop of the receptor binding site (RBS) are highlighted.

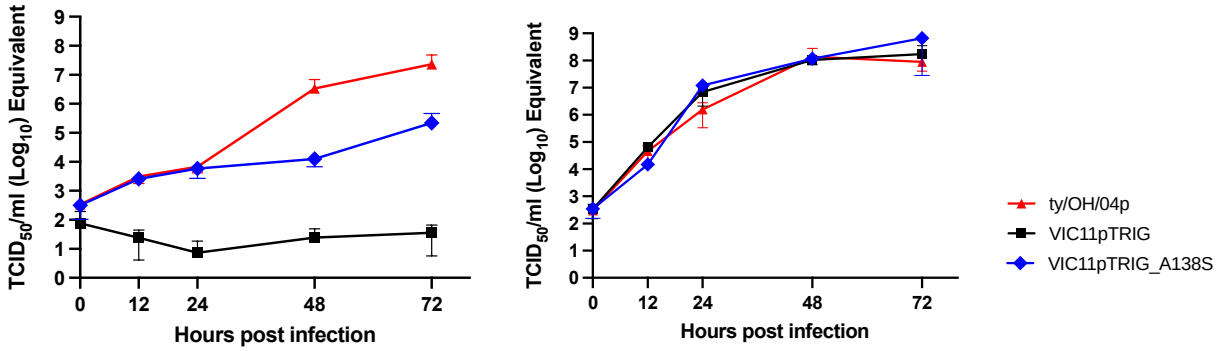

Figure 4.5. *In vitro* replication kinetics of mutant containing A138S substitution in primary swine tracheal epithelial cells (pSTECs) and MDCK cells. Viral growth kinetics of A138S mutant virus (VIC11pTRIG_ A138S), VIC11pTRIG reassortant and ty/OH/04p in (A) pSTECs and (B) MDCK cells. pSTECs and MDCK cells were infected at an MOI of 0.5, and supernatants were collected at 0, 12, 24, 48, and 72 h post infection (hpi). Viral titers were quantified by $TCID_{50}$. Values are shown as mean $TCID_{50}$/ml titers ± standard error of the mean.

Table 4.1. Lung and trachea microscopic pathology and virus detection in pigs challenged with reassortant viruses and negative controls[a]

Virus	Microscopic lung lesions score[b]	Lung IHC score[c]	Microscopic trachea lesions score[c]	Trachea IHC score[d]
NC	0.25 ± 0.16^{A}	0.0 ± 0.0^{A}	0.20 ± 0.20^{A}	0.0 ± 0.0^{A}
A/VIC/11	0.78 ± 0.19^{A}	0.0 ± 0.0^{A}	0.75 ± 0.22^{A}	0.0 ± 0.0^{A}
VIC11rg	0.60 ± 0.18^{A}	0.0 ± 0.0^{A}	0.20 ± 0.15^{A}	0.0 ± 0.0^{A}
VIC11p	0.58 ± 0.15^{A}	0.0 ± 0.0^{A}	0.05 ± 0.05^{A}	0.0 ± 0.0^{A}
VIC11pTRIG	0.48 ± 0.21^{A}	0.0 ± 0.0^{A}	0.65 ± 0.18^{A}	0.02 ± 0.02^{A}
sw/MO/14	9.43 ± 0.61^{B}	5.3 ± 0.4^{B}	2.13 ± 0.29^{B}	2.58 ± 0.23^{B}

[a] Results are shown as means ± standard errors of the means. *Different uppercase letters* within the same column *indicate significant difference at P<0.05.* [b] The range of possible scores is 1 to 22. [c] The range of possible scores is 1 to 8. [d] The range of possible scores is 1 to 4. IHC, immunohistochemistry.

Table 4.2. Variants identified in directly inoculated and respiratory contact pigs infected with VIC11pTRIG. List of variants in nasal swab samples from directly inoculated and respiratory contact pigs in this study. The numbers on the top represent the nucleotide positions. The VIC11 HA represents the HA sequence of A/Victoria/361/2011/H3N2, which is the same as VIC11pTRIG. Substitutions at the positions relative to the reference sequence present in ≥2% of variant viruses are shown. Number of samples containing each variant for either directly inoculated or respiratory contacts are listed. *Positions 489, 634, 655 correspond to amino acid residues 138, 186, 193, respectively.

	89	**199**	**256**	**286**	**296**	**489***	**553**	**563**	**585**	**634***	**640**	**643**	**655***	**711**	**730**	**742**
VIC11 HA	T	A	A	T	C	G	T	A	A	T	A	A	T	G	G	G
Variant Sequence	C	G	G	C	T	T	A	G	G	G	G	G	A	T	A	A
Directly Inoculated		1			2	**5**	1	**1**	1	**2**			**2**	2		
Contact	1		1	1		**9**		**1**		**2**	1	1	**2**		1	1

	748	**751**	**757**	**762**	**802**	**959**	**997**	**1025**	**1060**	**1109**	**1112**	**1144**	**1196**	**1244**	**1264**	**1271**
VIC11 HA	G	A	C	A	T	C	G	G	C	G	A	A	A	C	A	A
Variant Sequence	A	C	A	G	C	T	A	A	T	A	G	G	T	T	G	G
Directly Inoculated			1	1	2	1	1	1		1			1	1	1	1
Contact	1	1							1		1	1				

	1284	**1286**	**1335**	**1412**	**1418**	**1449**	**1487**	**1536**	**1601**	**1648**	**1686**	**1692**	**1722**	**1748**
VIC11 HA	G	A	C	C	G	A	C	G	G	G	T	T	T	A
Variant Sequence	A	G	T	T	A	G	T	A	A	A	G	C	C	C
Directly Inoculated	1	1	1			1	1	1	1	1	1		1	
Contact				1	1							1		1

CHAPTER 5

PASSAGE OF HUMAN H3N2 INFLUNEZA A VIRUS IN PRIMARY SWINE TRACHEAL EPITHELIAL CELLS SELECTS FOR ADAPTIVE MUTATIONS ON THE HA GENE THAT INCREASE REPLICATION AND FITNESS

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Abstract

Influenza A viruses (IAV) are enveloped viruses that can cross between swine and humans. Frequent spillover from humans, coupled with reassortment and antigenic evolution, contribute to the increased diversity of IAV circulating in pigs. Although human-to-swine spillover events are frequent and have contributed many lineages of endemic swine viruses, little is known about the adaptation process between the two species. To understand the evolutionary dynamics of human IAV in pigs, we examined the changes that occurred during serial passages of a reassortant H3N2 virus containing human seasonal surface genes (HA and NA) and a swine-adapted internal gene constellation in differentiated primary swine tracheal epithelial cells (pSTECs). The reassortant virus (VIC11pTRIG) was serially passaged 8 times in pSTECs and compared to a control, swine-adapted strain containing the same backbone of internal genes. Viral RNA from passages 0 (inoculum), 1, 3, 5 and 7 were sequenced using next generation sequencing (NGS) technology. Viral population diversity was highest at passage 3. We identified two amino acid mutations in the HA protein (N165K and N216K) that became dominant at passage 4 and fixed at passages 7 and 5, respectively. These changes were associated with increased fitness of the virus in pSTECs compared to the original reassortant strain containing wild type HA/NA. Our results suggest that adaptation of human seasonal H3N2 to swine cells is conferred by selection of HA mutations located near the receptor binding site.

Keywords: influenza A virus, human, swine, primary swine trachea epithelial cell, Air-Liquid-Interface, reverse genetics, virus evolution, serial passage

Introduction

Influenza A virus (IAV) is a member of the *Orthomyxoviridae* family that causes substantial economic burden to the swine industry. IAV infects several different animal hosts species, which play an important role in the dissemination and maintenance of the virus. IAV has the ability to cross between species; however, sustained transmission in the new host is rare [9, 148]. The surface proteins of IAV, hemagglutinin (HA) and neuraminidase (NA), display a high degree of variability resulting from two main mechanisms, antigenic shift and antigenic drift [158]. Although only H1N1, H3N2, and H1N2 subtypes circulate in pigs globally, significant genetic diversity is observed between and within subtypes [106, 159, 160]. This diversity is largely the result of transmission of IAV from other species, followed by antigenic shift and drift in the swine population [3, 5, 9, 13, 112, 118]. An important example of this evolutionary dynamics is the continuing circulation of the triple reassortant internal gene (TRIG) constellation, which was introduced in North America over two decades ago [161, 162]. Most of the swine IAV circulating in the U.S. still contain genes of the TRIG constellation [163]. The TRIG cassette was shown to stably incorporate HA and NAs from various origins, potentially explaining the increased evolutionary rate of swine IAV and increased diversity after its introduction in the U.S. [11, 160, 163].

The frequent bidirectional transmission between humans and swine has led to establishment of many novel virus lineages in pigs, contributing to the increased diversity of IAV circulating globally [3, 13]. Monitoring programs have frequently identified human-origin genes within IAV circulating in the U.S. swine population [15, 159, 164], often displaying changes in specific positions that can alter antigenicity and infectivity in

pigs [13, 14, 118]. However, the molecular mechanisms that allow human origin H3N2 IAVs to adapt to pigs are not well understood. Although the HA is recognized as a major determinant of host specificity due to its role in receptor binding [7], other viral proteins have also been shown to affect host specificity of IAV [52, 54] A previous study investigating the adaptation of human-origin H3N2 viruses to pigs showed that, although the HA was crucial, the internal gene constellation also played a role [13], suggesting the adaptation of IAV between humans and pigs is a multigenic trait. We have previously shown that a reassortant H3N2 virus (VIC11pTRIG) containing human seasonal HA and NA genes from human seasonal A/Victoria/361/2011 H3N2 and internal gene constellation from the TRIG and pdm09 lineages was able to transmit between pigs, while viruses containing human-origin IAV internal genes did not transmit [Chapter 4]. A critical mutation (A138S) was identified after a single transmission round during the study and shown to improve replication in primary swine tracheal epithelial cells (pSTECs) [Chapter 4]. These results indicate that the ideal constellation of internal genes is needed to allow for the surface gene segments to persist and evolve in pigs.

Differentiated cells grown using the Air liquid interface (ALI) [165] provide an ideal *in vitro* model that mimics the natural environment of the host as they retain key structures such as the pseudostratified epithelium, cilia, and mucous cells [166]. Differentiated cell cultures are commonly used for studying toxicology of the respiratory tract [165, 166], cancer, pharmacology and chemical research [167-169]. These in vitro models are also used to assess pathogenesis and replication of different pathogens such as influenza virus, respiratory syncytial virus, and bacterial infections [42, 84, 165, 170]. For this study, differentiated pSTECs were used to simulate long term evolution in pigs.

We evaluated the evolutionary dynamics for the adaptation of human seasonal HA and NA genes by consecutive passages in primary swine tracheal epithelial cells (pSTECs) using next generation sequencing (NGS). We have identified mutations in the HA gene that seem to improve replication in pSTECs.

Material and Methods

Methods used for this Chapter are described in detail in Chapter 3.

Results

Serial passage of VIC11pTRIG in primary swine tracheal epithelial cells (pSTECs) increased viral replication

Supernatants were collected from passages 1 to 8 (P1 to P8) after serial passages of VIC11pTRIG and ty/OH/04p in pSTECs and titrated by the $TCID_{50}$ method (Fig. 5.1). The viral titers of VIC11pTRIG were relatively low in early passages, from P1 to P2 with an average titer of 5.0×10^2 (Fig. 5.1A). However, titers gradually increased until reaching 1.2×10^5 $TCID_{50}$/ml in P4, remaining high in subsequent passages, with an average titer of 1.0×10^5 $TCID_{50}$/ml. At the last passage (P8), the virus titer was measured at 6.0×10^4 $TCID_{50}$/ml. In contrast, titers of ty/OH/04p were high from the start of the passage experiments, with viral titer of 8.6×10^6 $TCID_{50}$/ml in P1 and an average of 9.6×10^6 $TCID_{50}$/ml throughout P8 (Fig. 5.1B).

Serial passage of VIC11pTRIG in primary swine tracheal epithelial cells (pSTECs) resulted in the emergence of dominant variants

The inoculum and supernatants in passages 1, 3, 5, and 7 were sequenced by NGS and analyzed for HA nucleotide changes. A total of 12 variants present in ≥2% of viruses were observed in the HA and 7 variants in the NA of VIC11pTRIG, including a silent mutation at nucleotide position 385 (Table 5.1). For the ty/OH/04p strain, a total of 14 variants were discovered in the HA and 16 variants in the NA, including two silent mutations emerging at nucleotide positions 240, 538 at P7 in the HA (Table 5.2). Two HA variants, N165K and N216K, became dominant at P4 of VIC11pTRIG (confirmed by consensus Sanger sequencing) and became fixed at P5 (N216K) or P7 (N165K) (H3 numbering) (Table 5.1). The two dominant mutations were maintained until the last passage (P8). There were no dominant mutations in the HA of VIC11pTRIG at P1 and P3 and only the two silent mutations became dominant for ty/OH/04p (P7).

All major variants identified in the HA were located within the receptor binding sites (RBS) of the HA

The 2 dominant variants identified in the HA of VIC11pTRIG were located in the head domain, near the receptor binding site (RBS) (Fig. 5.2). The N165K was located near the 130 loop, but relatively far from the other RBS structures. The N216K mutation was located between the 130 loop, the 190 helix, and the 220 loop. The two mutations were also located in the H3 antigenic site B [151].

Mutant viruses show better replication in primary *swine tracheal epithelial cells (pSTECs) than VIC11pTRIG*

Mutant strains of VIC11pTRIG were generated and viral growth kinetics were evaluated in pSTECs and MDCKs to compare replication efficiency in relation to the original VIC11pTRIG containing wild type HA (Fig. 5.3A). Mutants were generated with single or double mutations. Overall, all mutants showed more efficient replication compared to the VIC11pTRIG. The double mutant (VIC11pTRIG_DM) showed the highest titers at 12 h post-infection (hpi), with titer of 1.2×10^5 $TCID_{50}$/ml, followed by VIC11pTRIG_N165K and VIC11pTRIG_N216K with titers of 1.1×10^4 $TCID_{50}$50/ml. The ty/OH/04p and VIC11pTRIG strains showed the lowest titers at this time-point. While the VIC11pTRIG strain titer dropped at 24 hpi, all viruses continued to grow, and remained higher than VIC11pTRIG. At 48 and 72 hpi, VIC11pTRIG_N216K and ty/OH/04p showed similar titers at an average of 8.5×10^6 $TCID_{50}$/ml titers followed by VIC11pTRIG_N165K and VIC11pTRIG_DM at an average of 3.4×10^5 $TCID_{50}$/ml, all significantly higher than VIC11pTRIG by at least 2 logs. In contrast, there were no significant differences observed among viruses in growth kinetics in MDCK cells (Fig. 5.3B).

IAV isolated from pigs globally show evolution in the 165 and 216 positions in recent years

To assess the residues at the 165 and 216 amino acid positions found in natural isolates detected in pigs, a total of 3181 HA protein sequences were obtained from the Influenza Research Database (IRD). In North American viruses, there was minimum variation between the years 2000 and 2014 (Fig. 5.4A). However, between the years 2015-2019, a large percentage of viruses containing Glutamine (E) at amino acid position

165 (N165E) started to be detected, becoming predominant in 2020, but proportions between the two residues (E and N) became similar in 2022. For viruses isolated from Asia, Europe, South America, and Oceania, little variation at the 165 amino acid position was observed between years 2000 and 2009, but viruses isolated between 2010-2014 showed similar proportions of Lysine (K) compared to Asparagine (N) at this amino acid position (N165K; Fig. 5.4B). In contrast, there was very minimum variation at the 216 amino acid position regardless of region where they were isolated, with 99% of viruses containing Asparagine (N; Fig. 5.4C,D).

Discussion

Human-to-swine spillover events are common, contributing to the large diversity of swine IAV circulating in pigs globally [5, 10, 147]. While the factors that allow human-origin IAV to adapt to pigs are not fully understood, the ideal combination of genes seems to be an important step. As an example, the circulation of the TRIG cassette may have contributed to the increased frequency in the establishment of human-origin IAV lineages in pigs, as it is permissive to varied HA and NA surface proteins [5, 13, 102]. We have previously shown that the TRIG cassette in combination with the H1N1pdm09 matrix gene formed an ideal backbone for transmission in pigs of surface genes of human origin [Chapter 4]. Here, we assessed the evolution of human-origin HA and NA within that same internal gene constellation during serial passages in differentiated pSTECs. The pSTECs are good surrogates for *in vivo* conditions and have many advantages over submerged cell cultures, as differentiated cells grown under ALI can develop pseudo-stratified epithelium with cellular features that resemble those from

in vivo environment [170].

During the serial passages of the human seasonal reassortant virus (VIC11pTRIG) in pSTECs, 2 dominant mutations emerged in the HA, N165K and N216K, at P4 and were maintained until the last passage (P8). These mutations were correlated with increased virus fitness as shown by the increased titers starting at P4 during serial passages and more efficient replication in viral growth kinetics. IAV has been shown to gain mutations during serial passages *in vivo* and *in vitro*, resulting in increased fitness and/or escape from the host immune system [171-175]. A recent study showed that serial passage of an avian H9N2 in pigs resulted in increased transmissibility in swine, and several mutations were acquired during the process, including mutations in the avian-origin internal genes [176]. In the current study, the early passages of VIC11pTRIG did not replicate very efficiently, likely because its human surface genes were not well adapted to the swine cells, but fitness improved once mutations N165K and N216K became dominant. We showed previously that a single mutation acquired during replication of the VIC11pTRIG in pigs contributed to increased respiratory transmission [Chapter 4]. Although in this study the 2 mutations that were fixed differed from the previous study performed in animals, all these mutations were located near the receptor binding site of the IAV HA. Differences between mutations may be due to differences in the systems used: here we used an *in vitro* system in which differentiated cells were derived from animals at 5 months of age (~20 weeks old), while the animals used in the *in vivo* study were 4 weeks old, likely incurring age-related individual variation. Additionally, although a very advantageous system that close resembles the animal's respiratory tract cells, differentiated ALI epithelial cells may lack intrinsic factors and

other immune cells that are involved during IAV infection. Hence, the reassortant human IAV would have to overcome additional bottlenecks during replication and transmission in the live swine host [Chapter 4]. Although both mutations (N165K and N216K) emerged in combination and were maintained for 4 passages, no additive effect was observed between them, as there was no major differences in replication kinetics among the N165K, N216K and double-mutant variants. Though both mutations showed a similar effect in viral fitness, we cannot disregard that one might have been a compensatory or a hitchhiker mutation for the other. This hypothesis deserves further investigation to assess which mutation, if any, exerts dominance.

The 165 position is one of the most conserved glycosylation sites in H3N2 IAV sequences [177] and has been shown to be involved in immune escape in humans [178]. N-glycosylations promote immune escape by physically preventing antibodies from binding to specific antigenic sites. In a similar way, N-glycosylations can also shield the receptor binding site and result in decreased binding to cell receptors [178, 179]. The N165K mutation in the A/VIC/11 HA leads to a putative glycosylation loss, which may have improved binding to swine cells. Perhaps related, loss of the glycosylation site at the 165 position was shown to increase replication of human H3N2 IAV in guinea pigs [180]. Interestingly, the number of glycosylations on the HA head has been shown to affect susceptibility to the antiviral activity of innate collectins such as surfactant protein D (SP-D) [181, 182]. Since porcine SP-D has been shown to inhibit IAV more efficiently than human SP-D, it is possible that this is an important step during infection of swine cells in which selective pressure is exerted on the human-origin HA. Residues in position 216 seem to be more conserved than in position 165. However, variations at the position were

shown to affect membrane fusion and pH regulation [183]. While the function of the position is not completely known, the mutation led to enhanced replication in pSTECs, to a similar degree of the 165 variant and the double mutant.

It is worth noting that little variation existed at position 165 in swine isolates detected globally until 2015, but variation increased after 2016. For the U.S., this increase in variability coincides with the establishment of the new 2010.1 H3N2 lineage in pigs [14], an HA lineage that has similar ancestry to the A/VIC/11 HA tested here. It is tempting to infer that our study recapitulates, to some degree, the evolution observed for position 165 as the novel human-origin 2010.1 virus was spreading in the swine population. Although the same amino acid change was not observed, both changes led to loss in glycosylation. Moreover, some viruses isolated from South America contained the N165K mutation observed here. We have identified potential positions that may have enabled adaptation of a virus containing human-origin surface genes to swine. Further studies are needed to evaluate the effect of these mutations *in vivo* and on subsequent virus evolution.

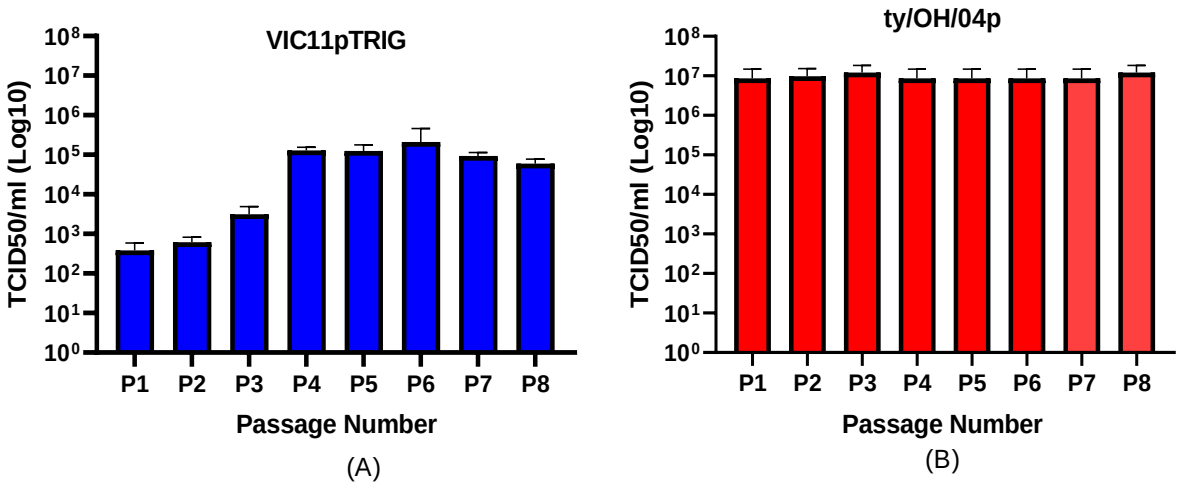

Figure 5.1. Viral titers in the supernatant collected from serial passages of reassortant VIC11pTRIG and ty/OH/04p influenza viruses in primary swine tracheal epithelial cells (pSTECs). A total of 8 serial passages were conducted in pSTECs to determine viral $TCID_{50}$/ml titers. Titers were calculated via the Reed–Muench method. Values are shown as mean $TCID_{50}$/ml titers ± standard error of the mean. (A) Titers of passage 1 to passage 8 (P1-P8) of VIC11pTRIG virus. (B) Titers of P1-P8 of ty/OH/04p virus.

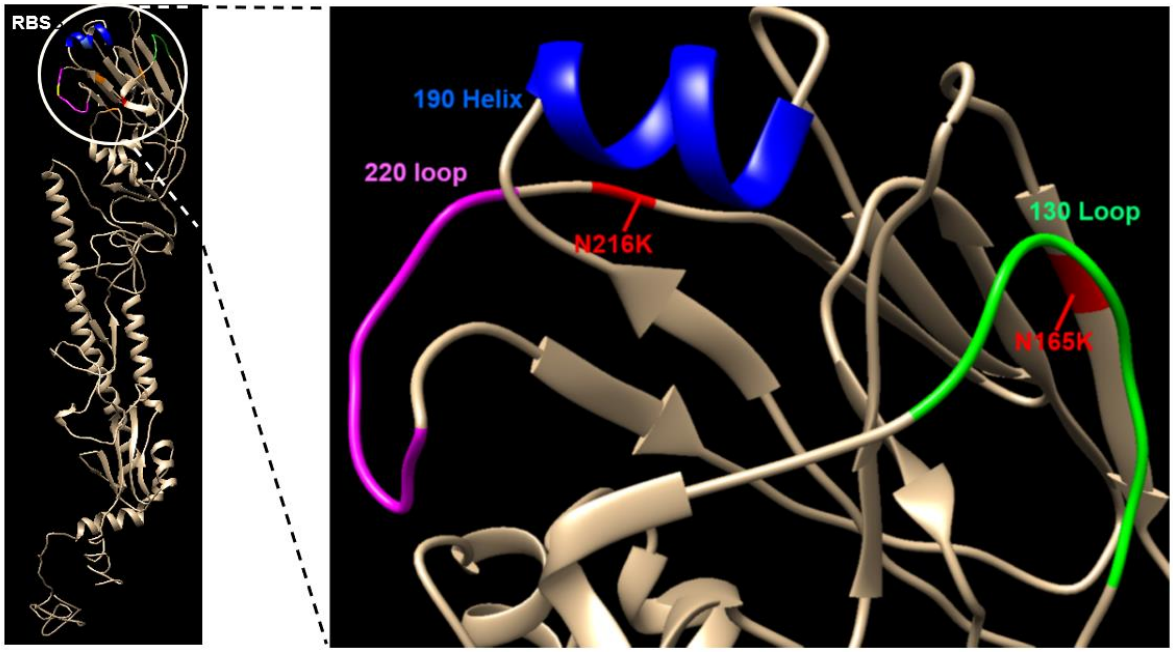

Figure 5.2. Location of mutations identified on the Vic11pTRIG HA. Dominant variants identified in this study on the HA of the VIC11pTRIG are highlighted on the structure of the human A/Victoria/361/2011 H3. Red, residues N165K and N216K. The 190 helix, 130 loop, and 220 loop of the receptor binding site (RBS) are highlighted.

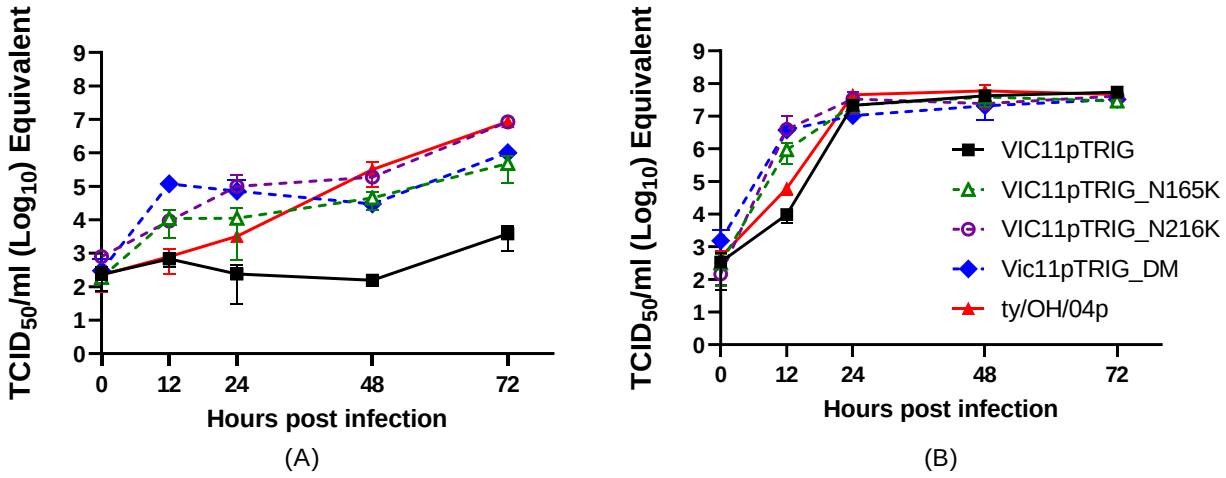

Figure 5.3 *In vitro* replication kinetics of mutants containing N165K and/or N216K substitutions in primary swine tracheal epithelial cells (pSTECs) and MDCK cells. Viral growth kinetics of mutant viruses (VIC11pTRIG_ N165K, VIC11pTRIG_N216K, VIC11pTRIG_DM), VIC11pTRIG reassortant and ty/OH/04p in (A) pSTECs and (B) MDCK cells. pSTECs and MDCK cells were infected at an MOI of 0.05, and supernatants were collected at 0, 12, 24, 48, and 72 h post infection (hpi). Viral titers were quantified by rtRT-PCR and titers calculated by $TCID_{50}$ equivalency. Values are shown as mean $TCID_{50}$/ml equivalent titers ± standard error of the mean.

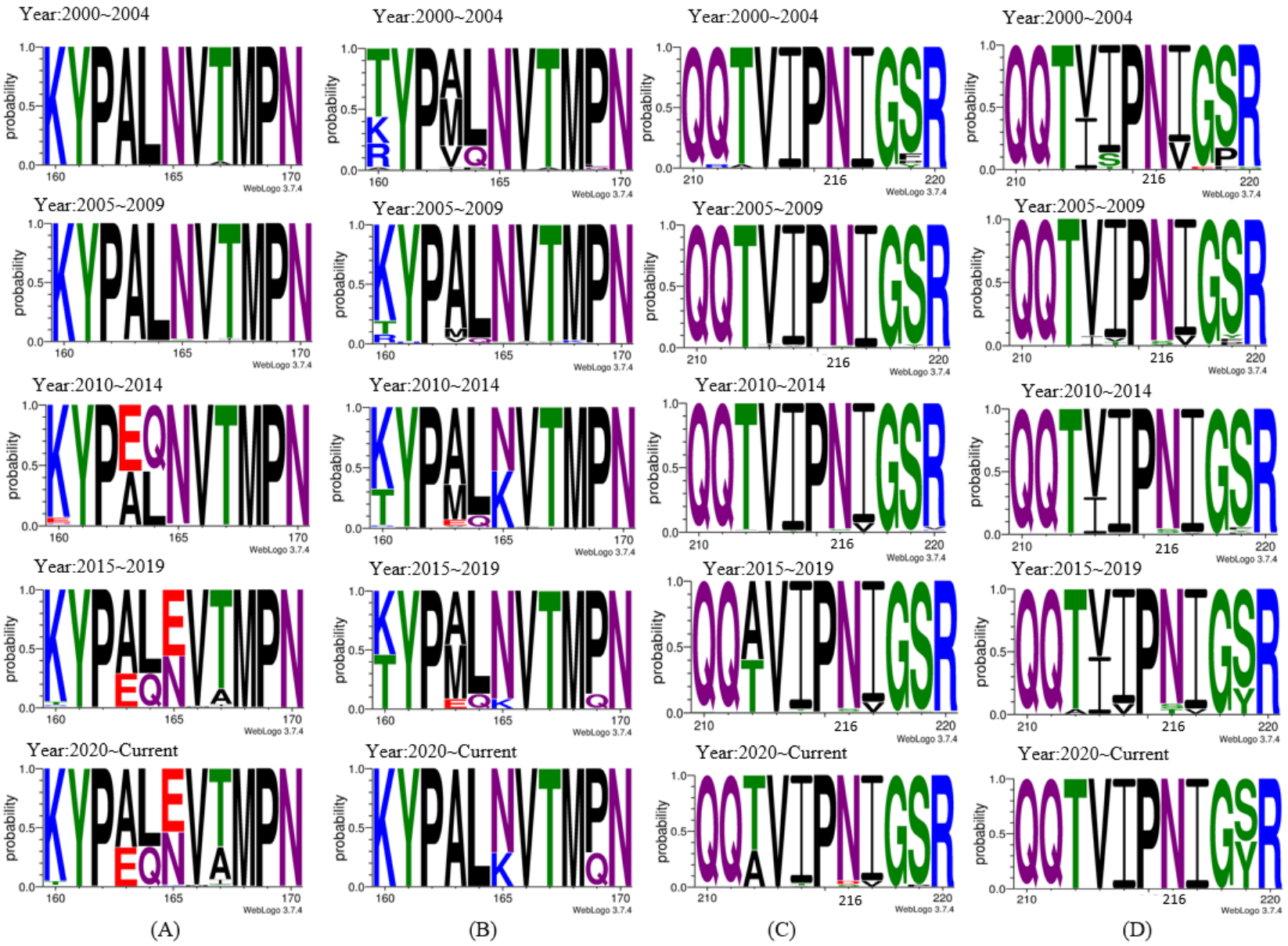
Year:2000~2004
Year:2005~2009
Year:2010~2014
Year:2015~2019
Year:2020~Current
probability
WebLogo 3.7.4
(A)
(B)
(C)
(D)

Figure 5.4. Amino acid substitutions at position 165 and 216 of HA from H3N2s isolated in the world between year 2000~2020. The relative proportion of mutations between residues 161 and 170 position and 210 and 220 are shown in the figure, staggered for each 5 years. (A) Variations in position 165 in swine H3N2 IAVs isolated between 2000-2022 in North America (total of 2,928 HA protein sequences). (B) Variations in position 165 in swine H3N2 IAVs isolated between 2000~2022 in Asia, Europe, South America and Oceania (total of 353 HA protein sequences). (C) Variations in position 216 in swine H3N2 IAVs isolated between 2000-2022 in North America. (D) Variations in position 216 in swine H3N2 IAVs isolated between 2000~2022 in Asia, Europe, South America and Oceania. Sequences were obtained from Influenza Research Database (IRD) [145] and logos created with Weblogo version 2.8.2 [146].

Table 5.1. Variants identified during serial passage of VIC11pTRIG in primary swine tracheal epithelial cells (pSTECs). List of variants identified during serial passages in this study. Nucleotide mutations present in ≥2% of variant viruses are shown. The numbers between parenthesis represent amino acid mutations, followed by frequency of detection by NGS. All mutations were confirmed by NGS or Sanger sequencing. P1-P8 represent passages 1 to 8.

Passage	HA	NA
P1[a]	g900a (0.03), g1044a (0.03) a1447g (0.04), g1545a (0.04)	t149c (0.05), a267c (0.05) g680a (0.02), g1150t(0.09) g1158c (0.1), g1375g (0.05)
P2	n/a	n/a
P3[a]	t836c (0.03), t1121c (0.03), t1473g (0.04) g915a (0.05), t922a (0.06) c1466a (0.02)	**c1174t (385N, 0.99)**[c]
P4[b]	**c572a (N165K), t725a (N216K)**	n/a
P5[a]	**c572a (N165K, 0.95), t725a (N216K, 0.99)**	N
P6[b]	**c572a (N165K), t725a (N216K)**	n/a
P7[a]	**c572a (N165K, 0.99), t725a (N216K, 0.99)**	N
P8[b]	**c572a (N165K), t725a (N216K)**	N

[a]Mutations confirmed by NGS. [b]Mutations confirmed by Sanger sequencing. [c]Silent mutation

Lower case letters, nucleotides; upper case letters, amino acids; bold, dominant mutations. N-No mutations or variants (≥2%) were detected. n/a-non-available

Table 5.2. Variants identified during serial passage of ty/OH/04 in primary swine tracheal epithelial cells (pSTECs). List of variants identified during serial passages in this study. Nucleotide mutations present in ≥2% of variant viruses are shown. The numbers between parenthesis represent amino acid mutations, followed by frequency of detection by NGS. All mutations were confirmed by NGS or Sanger sequencing. P1-P8 represent passages 1 to 8.

Passage	HA	NA
P1[a]	g1586t (0.03)	g703a (0.1), g1205a (0.04) g1403a (0.05)
P2	n/a	n/a
P3[a]	a1233g (0.03), g744a (0.02) g748a (0.03)	g730a (0.04), g1403a (0.04), g1405a (0.02)
P4	n/a	n/a
P5[a]	c418t (0.021). g1116a (0.02) g1536a (0.02), g1241a (0.02)	g392a (0.032), a899g (0.02) g951a(0.07), g1126a (0.04), g1370a (0.02), g1405a (0.04)
P6	n/a	n/a
P7[a]	**g748a (240R, 0.57)[c], t1642c (538I, 0.55)[c]** c418t, c755a (0.02), a1230g (0.02), a1233g(0.32)	g645a (0.026), a815g(0.04), a1122g (0.04), g1126a (0.45)
P8[b]	N	n/a

[a]Mutations confirmed by NGS. [b]Mutations confirmed by Sanger sequencing. [c]Silent mutation

Lower case letters, nucleotides; upper case letters, amino acids. N-No mutations or variants (≥2%) were detected. n/a-non-available

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CHAPTER 6

CLOSING REMARKS AND FUTURE PROSPECTS

Swine influenza is an important disease which poses a significant threat to pigs and human alike, in terms of economic impact on the pig industry and public health. The diversity of swine IAVs are steadily increasing, and human-to-swine spillover events are common. In order to effectively combat and prevent the spread of swine IAVs within the pig population, biosecurity measures such as implementing proper animal management practices and developing vaccines for both human and pigs is critical. However, such practices require accurate knowledge of how the viruses spread and potential prediction of how they will evolve in the future. While surveillance is instrumental in painting the current picture on how swine IAVs are evolving, accurate prediction of how the viruses evolve is problematic at best. While frequent human-to-swine spillover events occur, and human-origin segments are present in most swine IAV isolates, the exact amino acid signatures which enables the human-origin H3N2s to adapt to swine is not clearly determined. This dissertation aimed to investigate the molecular changes that are required for human H3N2 viruses to transmit, replicate and adapt in pigs. Thus, the first stage was to determine the ideal constellation of gene segments which would readily transmit and adapt to pigs. We tested three different internal gene backbones, one of human origin, one of pandemic origin and one of a mixture between TRIG and pandemic origin. While

both the TRIG and pandemic backbone contributed to efficient replication and transmission in pigs, the strain with the TRIG/pdm cassette was the only strain containing whole human origin viral surface genes that was able to transmit between pigs.

As shown in Chapter 4, acquiring an ideal backbone is the first step for a human-origin virus to result in transmission in pigs. The human virus containing the pandemic M gene segment showed higher replication with a greater number of pigs being infected than its counterparts which contain wholly human-origin gene segments, highlighting the swine-adapted M gene may also contribute to replication. Coupled with the fact that the TRIG lineage is part of the most prominent constellations currently circulating in the United States, this dissertation provided strong evidence that the human origin viruses with the TRIG/pdm cassette have contributed to the establishment of human-origin IAV gene segments in pigs. During the respiratory contact study in Chapter 4, a dominant HA variant, containing amino acid mutation A138S, was shown to become fixed in contact pigs. The surface genes of IAV are subject to evolution and the HA shows the highest mutation rate. To check if the A138S affected the fitness of the virus, viral kinetics were performed, showing the mutation increased replication in primary swine tracheal epithelial cells (pSTECs). Given that the mutation was located near the Receptor Binding Site (RBS) of the HA, it is possible to speculate the mutation may have induced conformational change on the HA, enabling the human-origin HA to improve binding and successfully transmit and adapt to pigs.

Following these studies, we utilized differentiated pSTECs to emulate the natural *in vivo* environment and conducted serial passages to evaluate the long-term evolution of the human-origin HA during the adaptation process. Differentiated cells are widely used

as surrogates for *in vivo* experiments in infection studies or pharmacology studies as they retain most of the structures that resemble those of *in vivo* organs. Two mutations, N165K and N216K, emerged during serial passage and were maintained until the end of the study. Variant analysis confirmed these mutations were fixated after passages 7 and 5, respectively. The mutations emerged at passage 4 which is also the same passage when significant increase of viral titers were observed, indicating the mutations were instrumental in increasing replication and fitness of the virus. This was confirmed in growth kinetic studies, when mutants containing the N165K and N216K mutations showed better replication than the original VIC11pTRIG virus. Thus, all the mutations identified in this dissertation, A138S N165K and N216K were shown to increase fitness of the VIC11pTRIG reassortant virus, indicating these may be evolutionary pathways for human-origin viruses to adapt to pigs.

All the mutations were located in the RBS of the HA, which supports change in the HA must occur for a virus to adapt to a new host. However, despite the similar location, the mutations that emerged in the *in vivo* studies differed from those in the *in vitro* studies. The difference may indicate initiation of two separate evolutionary pathways during the process to achieve complete host adaptation providing they emerged as a product of positive selection. However, the different parameters between the studies must be considered. In the animal study, the VIC11pTRIG reassortant virus would have been faced with two major bottlenecks- the reduction of diversity commonly known to happen during transmission, while at the same time encountering the new host's immune response that could potentially reduce diversity further. This is different from the serial passage studies, in which the reassortant would have faced a more relaxed bottleneck as

adequate number of viral particles were carried over for each passage in differentiated pSTECs. Also, while differentiated tracheal cells are known to possess a certain level of immunity, the pressure it exerts on the reassortant would be somehow more lenient compared to the response that an animal's immune system can mount. The N165K mutation observed here resulted in loss of glycosylation, which may decrease susceptibility against the innate immune response. Hence, the variant was likely more resistant to replicate and adapt to the swine origin cells. However, this hypothesis needs to be evaluated by further studies.

Once amino acid signatures that increase fitness and replication were identified, the next step would be to investigate if the mutations induced conformational change in the HA which may enable increased binding against the swine sialic acid (SA) receptors, given they were located within the RBS. One way to investigate would be modeling the binding abilities of the HA via protein interaction-docking assays. If any of the mutations affected the affinity of the HA, it would form a stronger bond in terms of molecular interactions, such as increase in H-bonds or the strength of Van de vaals bonds. Creating in-silico mutants and testing them with modeled swine SA receptors would be a way to investigate these types of interactions. Additionally, modeling other amino acids on the positions may provide some degree of prediction on what mutations may occur on the site, given it affects binding affinity.

Another step would be studying the nature of the mutations *in vitro*. Use of glycan assays would greatly help understand how the variants interact with the host cell beyond the sialic acid linkages. It is well-known that binding of the HA to host cells involves much more than the traditional SA-linkages. Development of synthetic chemistry have

lead to production of various glycan reagents allowing these kinds of assays to be readily available. Lectin binding assays would be a good method to characterize glycosylation, as there was a mutation which resulted in loss of glycosylation. Comparing the original VIC11pTRIG and the N165K variant would provide some explanation on the loss of glycosylation. Furthermore, these kinds of assays targeting cellular receptors can be expanded to study the difference of recognition patterns among viruses, especially between swine and human viruses. Swine and human viruses are known to recognize different types of neuraminic acid family(Neu) which includes sialic acids. Deeper analysis of these sialic acid molecular species would provide insight on the binding affinity of these viruses in different hosts.

The implications of the effect that the variants may have on immunity would be an important aspect that warrants investigation. Evaluating how the mutations would affect the immune escape ability of the virus would be relatively easy, as it would involve generation of anti-sera against the original VIC11pTRIG strain (A/VIC/11 HA) and testing it against all the mutants identified in the dissertation. It would provide extra insight on the antigenicity of the mutations. Since there is a possibility that the innate immune response may be one of the factors for the different mutations that emerged during the *in vivo* and *in vitro* studies, studying the immune response in both models would be important. For this purpose, a baseline to quantify immune response in both animal and differentiated cells would have to be established. Screening assays may help identify major immune factors that is consistent in both models and correlation with these factors and the immune pressure that is exerted against the virus would provide insight on how the immune factors played a role in the evolution of the strains.

Overall, this dissertation successfully identified mutations that increased replication and enabled transmission in pigs. Further studies must follow to determine the exact nature and define phenotypic changes of these mutations more in depth.