

## SWINE HEALTH

**Title:** Evaluation of novel reassortant swine influenza viruses (#12-095)

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### Industry Summary

Since the 2009 pandemic H1N1 virus (pH1N1) was found to transmit to pigs worldwide including in the USA, subsequent reassortment events between pH1N1 and endemic swine influenza viruses (SIVs) have occurred. We have isolated and characterized 60 swine influenza isolates from diseased or dead pigs in Midwestern swine farms with outbreaks of respiratory disease that include 1 H1N1, 11 H3N2 and 15 H1N2 reassortant SIVs containing 1 to 6 genes from the pH1N1. To date little is known about these novel reassortant SIVs and whether these novel reassortant viruses will be maintained in the swine operations. In the present proposal, we investigated pathogenicity and transmissibility of these novel reassortant viruses in pigs to determine whether they can be established and become predominant viruses in pigs.

To determine pathogenicity and transmissibility of novel reassortant H3N2 SIVs, 3 reassortant H3N2 and 1 endemic H3N2 viruses, which were full-genome sequenced, were selected for in vitro and the pig study: one reassortant H3N2 virus has 5 genes (PB2, PA, NP, M and NS) from the pH1N1; another 2 reassortant H3N2 viruses both contain NP, M and NS genes from the pH1N1, but they have a genetically different NA gene; the endemic H3N2 virus is a triple reassortant virus with genes from avian- (PB2 and PA), human- (HA, NA and PB1) and swine-origin (NP, M and NS) viruses. All 3 novel reassortant H3N2 viruses grew to higher titers than

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the control endemic H3N2 SIV in canine, swine and human cell lines. In the pig study, all 3 novel reassortant viruses were able to replicate efficiently in lungs and transmit to sentinel animals, similar to the control endemic H3N2 virus. The novel reassortant viruses with 3 genes (NP, M and NS) from pH1N1 were more transmissible when compared to the reassortant virus with 5 genes (PA, PB2, NP, M and NS) from pH1N1. Concurrent swine surveillance for influenza viruses showed that the novel H3N2 virus with 3 genes from pH1N1 is continually isolated from swine herds in the Midwest swine farms.

To investigate pathogenicity and transmissibility of novel reassortant H1N2 SIVs, One reassortant H1N2 (2+6 rH1N2), 1 H1N2 variant isolated from swine (swH1N2v), 1 H1N2 variant isolated from human (huH1N1v) and 1 endemic H1N2 (eH1N2) viruses were selected for the pig study: the selected reassortant H1N2 (2+6 rH1N2) virus has 6 internal genes (PA, PB2, PB1, NP, M and NS) from the pH1N1 and 2 surface gene from endemic H1N2 SIVs; the H1N2 variants isolated from swine (swH1N2v) and human (huH1N1v) have M gene from the pH1N1 and remaining 7 genes from endemic H1N2 viruses. All 4 viruses replicated efficiently in pigs' lungs and successfully transmitted to sentinel animals. However, both variant H1N2 viruses caused more severe lung lesions in infected pigs when compared to the 2+6 rH1N2 and eH1N2 viruses. Although all four viruses were detected in the lungs of contact animals, the swH1N2v shed more efficiently than the other three viruses in contact animals. Additionally, the huH1N2v displayed delayed and inefficient shedding kinetics in sentinel animals. Taken together, the H1N2 variant viruses are pathogenic and transmissible in pigs and could pose a threat to public and animal health.

All these results demonstrate that novel reassortant SIVs including H3N2 and H1N2 subtypes are pathogenic and transmissible in pigs which is in agreement with outbreaks observed in the swine farms. Concurrent swine surveillance for influenza viruses showed that both the novel H3N2 virus with 3 genes from pH1N1 and the H1N2 variant are 2 major novel reassortant viruses that are continually isolated from swine herds in the Midwest swine farms, indicating that they will be most likely maintained in swine herds and could pose a big threat to the swine industry.

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### **Scientific abstract**

Introduction of the 2009 pandemic H1N1 (pH1N1) virus into swine herds has resulted in reassortant events with endemic swine influenza viruses (SIVs) worldwide. We have isolated reassortant H3N2 and H1N2 SIVs containing 1 to 6 genes from pH1N1 from diseased pigs in U.S. Midwest swine farms with outbreaks of respiratory disease. However, little is known on the pathogenicity and transmissibility of these reassortant viruses in pigs and whether these novel reassortant viruses will be maintained in the swine operations. In the present study, we investigated pathogenicity and transmissibility of these novel reassortant viruses in pigs and performed concurrent syndromic surveillance to determine whether they can be established and become predominant viruses in pigs. Pig studies showed that all tested reassortant SIVs including H3N2 and H1N2 viruses are pathogenic and transmissible in pigs when compared to the endemic H3N2 and H1N2 viruses. Furthermore, the reassortant H3N2 viruses with NP, M and NS genes from pH1N1 are more transmissible in pigs when compared to the reassortant H3N2 virus with 5 genes (PA, PB2, NP, M and NS) from pH1N1; the H1N2 variant caused more severe lung lesions in infected pigs when compared to the reassortant H1N2 with 6 genes (PA, PB1, PB2, NP, M and NS) from pH1N1 and endemic H1N2 viruses. Concurrent swine surveillance for influenza viruses showed that both novel reassortant H3N2 virus with 3 genes from pH1N1 and the H1N2 variant are 2 major novel reassortant viruses that are continually isolated from swine herds in the Midwest swine farms. All these data indicate that both virus most likely will be maintained in swine herds and could pose a big threat to the swine industry.

### **Introduction**

Swine influenza is one of the most important respiratory diseases in pigs with high morbidity (approaching 100%) and low mortality (< 1%) rates. Despite the low mortality in swine herds, swine influenza is an economically important infectious disease for the swine industry. Since the first triple reassortant H3N2 swine influenza virus (SIV) was isolated from the US pigs in 1998, the triple reassortant H3N2 virus has been one of

the major subtype SIVs, which is circulating in pigs and continues to evolve through genetic mutation and reassortment with classical H1N1 and human H1 viruses forming novel reassortant H1 viruses. Fast evolution of SIVs and emergence of novel viruses has been an enormous challenge for producing efficacious vaccines to prevent and control swine influenza. Although commercial and autogenous vaccines were and are used in the US swine herds, swine influenza is still not efficiently controlled and has become an endemic and emerging disease in swine, resulting in significant economic losses for the swine industry each year.

Although the 2009 flu pandemic is relatively mild when compared to the last 3 pandemics in human history, the 2009 pandemic H1N1 virus (pH1N1) has the ability to cross the species barrier and has been detected from different species including dog, turkey, cat and swine. Since the first report on infection of pigs with the pH1N1 in Canada in 2009, pH1N1 has been isolated from pigs throughout the world including the USA. Introduction of pH1N1 into swine has raised concerns that novel reassortant viruses might be generated and become established in pigs which are more virulent and transmissible among pigs than the parental viruses. Indeed, several reassortments of SIVs with pH1N1 in swine have been reported in Asian and European countries. The first reassortant H1N1 virus was found in Hong Kong, China in 2009, which contains NA from pH1N1, HA from the Eurasian avian-like H1, and six internal genes from triple reassortant SIVs (Vijaykrishna et al., 2010). Subsequently, a reassortant H1N1 virus consisting of 7 genes from pH1N1 and NA from endemic SIVs was isolated in pigs in Germany (Starick et al., 2011). In early 2010, 3 reassortant H1N1 viruses were isolated from pigs in Thailand, which have NA from endemic H1N1 SIV and the remaining 7 genes from pH1N1 (Kitikoon et al., 2011). Two reassortant H1N2 viruses were isolated from pigs in UK and Italy respectively; one contains 6 internal genes of pH1N1 and another isolate has HA and 6 internal genes of pH1N1 and the remaining genes from endemic SIVs (Howard et al., 2011; Moreno et al., 2011). In the USA, nine H1N2, one H3N2 and one H1N1 reassortant SIVs have been detected in swine herds from Indiana, Minnesota and North Carolina. All these reassortant viruses contain the M gene and additional 1 to 4 internal genes from pH1N1, and the remaining genes from endemic triple reassortant SIVs (Ducatez et al., 2011; Sun et al., 2011). The reassortant H3N2 virus containing PA, NP and M genes from pH1N1 was tested in a ferret model and compared to a

pH1N1 and an endemic H3N2 virus. The results showed that the reassortant H3N2 virus replicated in ferret to the same extent as did the pH1N1 and endemic swine virus (Ducatez et al., 2011).

We have isolated 7 reassortant H3N2 SIVs having internal genes from pH1N1, 5 endemic triple reassortant H1N1, 4 endemic triple reassortant H1N2 and 3 endemic triple reassortant H3N2 SIVs from swine farms in the Midwestern USA with outbreaks of respiratory disease by working with a local veterinarian Dr. Steve Henry and Dr. Dick Hessen from Kansas State University Diagnostic Laboratory. These novel reassortant H3N2 SIVs were isolated between winter 2010 and spring 2011 from 5 swine farms in Kansas, USA which suffered outbreaks of respiratory disease (unpublished data). During the outbreak, pigs showed respiratory signs, such as coughing, sneezing, or mouth breathing in some animals. The morbidity was high (> 60%) and the mortality was low (< 3%) in the affected herds. The disease persisted in the swine herds throughout the winter in all affected farms. Primary sequence analysis showed that these 7 novel H3N2 SIVs were generated by reassortment between pH1N1 and endemic H3N2 SIVs and they can be divided into 3 genotypes which have a different genetic constellation when compared to one reassortant H3N2 virus reported previously (Ducatez et al., 2011). In summer and fall 2011, we continued to isolate novel reassortant H3N2 and also novel reassortant H1N2 SIVs with genes from pH1N1 from these herds, indicating that these reassortant viruses are still circulating and continue to evolve in the affected herds. The pathogenicity and transmissibility of these novel reassortant viruses in pigs, and whether these novel viruses will be maintained and become predominant viruses in swine herds remains unknown. Their potential threats to the swine industry will be investigated in this proposal.

## **Objectives**

The overall aim of this project is to evaluate the potential threat of novel reassortant swine influenza viruses (SIVs) containing genes from 2009 pandemic H1N1 virus to the US swine industry. The project aims to evaluate pathogenicity and transmissibility of novel reassortant SIVs with genes from the 2009 pandemic H1N1 virus (pH1N1) and to determine which genotype of these novel reassortant viruses might be maintained and prevail in swine herds.

Specific objectives:

- a. To sequence and characterize novel reassortant swine influenza viruses.
- b. To evaluate pathogenicity and transmissibility of novel reassortant viruses with genes from 2009 pH1N1 viruses in pigs.
- c. To determine which genotype virus will be maintained and prevail in swine herds.

## **Materials & Methods**

### *Molecular characterization of novel reassortant and endemic SIVs*

We will sequence the full genome of each novel reassortant SIV and characterize each novel genotype in vitro (different cell lines) and in vivo (pig model) to evaluate pathogenicity and transmissibility of these novel viruses. Molecular characterization and phylogenetic analysis will be performed for each gene segment. BLAST (<http://blast.ncbi.nlm.nih.gov>) and phylogenetic tree (MegAlign software version 4.1) analysis will be conducted to determine the source of the individual genes from the isolates. So far, three genotypes of novel reassortant H3N2 and 1 novel reassortant H1N2 viruses have been identified and will be selected for the pig experiments compared to one endemic H3N2 and one endemic H1N2 virus.

### *Pathogenicity and transmissibility of novel reassortant and endemic SIVs in pigs*

Experiment-1: Eighty one 3-4 week-old SIV- and porcine reproductive and respiratory syndrome virus- seronegative pigs will be allocated into 7 groups (12 pigs in each infected group and 9 pigs in control group). Nine pigs of each infected group and 9 control pigs will be intratracheally inoculated with  $10^6$  TCID<sub>50</sub> of each virus or virus-free MEM. Clinical signs and rectal temperature will be monitored daily during the course of the experiment for each pig. Three contact pigs will be comingled with the infected pigs in each group on 2 dpi to investigate viral transmission. Three infected and 3 control pigs will be necropsied on 3, 5 and 7 dpi, and 3 contact pigs will be euthanized on 4 dpc. Nasal swabs will be collected from infected pigs on 0, 3, 5, 7 dpi and from contact animals on 0, 3 and 5 dpc. Blood samples will be collected from all pigs before challenge and at the time of necropsy. During necropsy the lungs will be removed *in toto* from pigs. The percentage of gross lesions on each lung lobe will be scored by a single experienced veterinarian. Bronchioalveolar fluid (BALF) will be obtained by rinsing each lung with 50 mL MEM, viral titers of BALF and nasal swabs will be determined in MDCK cells. The right cardiac lung lobe will be collected and fixed in 10% buffered formalin, then stained with hematoxylin and eosin for histopathologic examination.

Viral load in BALF will be determined in a 96-well plate as described previously (Richt *et al.*, 2003). Briefly, 10-fold serial dilutions of each sample will be made in serum-free MEM supplemented with TPCK-trypsin and antibiotics. Each dilution (100  $\mu$ l) will be plated on PBS-washed confluent MDCK cells in 96-well plates. Plates will be evaluated for cytopathic effects after 24 to 72 h. At 72 h, plates will be fixed with 4% phosphate-buffered formaldehyde and immunocytochemically stained with a monoclonal antibody to influenza A nucleoprotein. The TCID<sub>50</sub>/ml will be calculated for each sample by the method of Reed and Muench. Virus will be isolated from nasal swab samples stored at -80°C by thawing and vortexing each sample for 15 sec, centrifuging it for 10 min at 640 x g, and passing the supernatant through 0.45- $\mu$ m filters to reduce bacterial contamination. An aliquot of 100  $\mu$ l will be plated on confluent, PBS-washed MDCK cells in 48-well plates. After incubation for 1 h at 37°C, 500- $\mu$ l serum-free MEM supplemented with 1  $\mu$ g/ml TPCK trypsin and antibiotics will be added. All wells will be evaluated for cytopathic effects after 48–72 h. Subsequently, plates will be fixed with 4% phosphate-buffered formaldehyde and stained as described above.

For histopathologic examination, lung sections will be examined by a veterinary pathologist in a blinded fashion and given a score of 0 to 3 to reflect the severity of bronchial epithelial injury (Richt *et al.*, 2003) according to the following criteria: 0.0: no significant lesions; 1.0: a few airways showing epithelial damage and light peribronchiolar lymphocytic cuffing, often accompanied by mild focal interstitial pneumonia; 1.5: more than a few airways affected (up to 25%), often with mild focal interstitial pneumonia; 2.0: 50% of airways affected, often with interstitial pneumonia; 2.5:  $\approx$ 75% of airways affected, usually with significant interstitial pneumonia; 3.0: >75% of airways affected, usually with interstitial pneumonia.

Experiment-2: Twenty 3-4 week-old SIV- and porcine reproductive and respiratory syndrome virus- seronegative pigs will be used in this study. Three novel reassortant SIVs and 1 endemic SIV will be selected based on the results of the experiment-1, which are highly pathogenic and transmissible among pigs. Three pigs will be intratracheally inoculated with 10<sup>6</sup> TCID<sub>50</sub> of each virus and these animals will be cohoused in a room. On 1 dpi, 6 sentinel pigs (contact group 1) will be comingled with the primary inoculated pigs. All primary infected pigs will be necropsied on 5 dpi and 2 sentinel pigs will be euthanized on 4, 6 and 8 dpc. Two pigs (contact group 2) will be comingled with the remaining 4 pigs in the contact group 1 on 5 dpi. The two pigs from the contact group 2 will be euthanized on 4 dpc. Nasal swabs will be collected from sentinel pigs of the contact group 1 on 0, 4, 6 and 8 dpc, and from pigs of the contact group 2 on 0 and 4 dpc. BALF samples will be collected from all experimental animals on the necropsy day. Virus in nasal swabs and BALF

samples will be titrated as described in experiment-1. The virus will be isolated from the nasal swabs and BALF samples and purified twice by plaque assays, and then amplified in MDCK cells. RNA will be extracted from the amplified viruses and RT-PCR will be performed to amplify each gene which will be sequenced to determine which virus (es) is in the respective samples. This analysis will inform us which genotype virus (es) will be most likely maintained in pigs.

## **Results:**

### *Molecular characterization of field isolated swine influenza viruses*

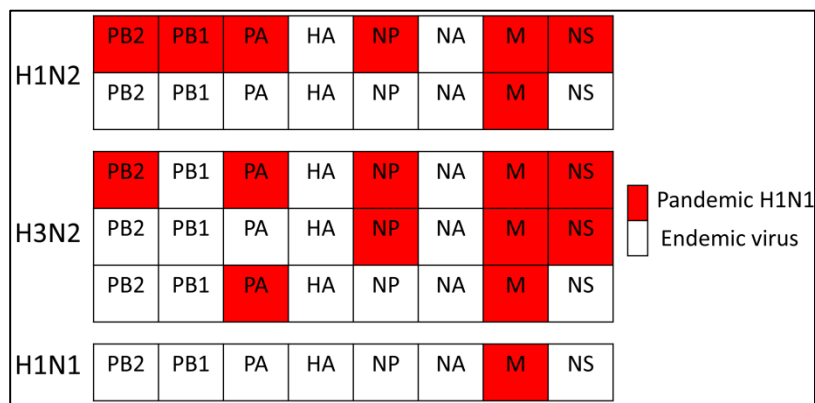
Previous and our studies showed that all novel reassortant SIVs have an M gene from the pH1N1 virus. Real-time PCR targeting on pH1N1 M gene was performed to screen the reassortant virus. We have sequenced and characterized 60 SIVs (partial or full genome) including 18 H1N1, 19 H3N2 and 23 H1N2 viruses that were isolated in Kansas swine farms (Table 1). For 23 H1N2 viruses, 15 viruses were confirmed to be reassortant viruses that contained only M gene or 6 internal genes (PB2, PB1, PA, NP, M and NS) from the pH1N1 and were full-genome sequenced (Table 1, Figure 1); 8 viruses were non-reassortant and their HA, NA and M genes were sequenced (Table 1). Eleven H3N2 SIVs were identified to be reassortant viruses with 2 (PA and M, 1 strain that was recently isolated), 3 (NP, M and NS, 9 isolates) or 5 (PB2, PA, NP, M and NS, 1 isolate) internal genes from the pH1N1 virus and were full-genome sequenced (Table 1, Figure 1); 8 H3N2 viruses were non-reassortant and 1 of 8 H3N2 non-reassortants were full-genome sequenced, and the HA, NA and M genes of remaining 7 isolates were sequenced. Seventeen of 18 H1N1 isolates were identified to be non-reassortant viruses, and their HA, NA and M genes were sequenced (Table 1); 1 H1N1 virus was a reassortant virus containing only M gene from pH1N1 (Figure 1). In Summary, 27 novel reassortant (11 H3N2, 1 H1N1 and 15 H1N2) and 1 endemic H1N2 and 1 endemic H3N2 viruses have been full-genome sequenced, and the HA, NA and M genes of 32 non-reassortant viruses were sequenced.



**Table 1: swine influenza viruses isolated from Kansas swine farms**

	Isolate No.	Reassortant No.	Non-reassortant No.
H1N1	18	1	17
H1N2	23	15	8
H3N2	19	11	8
Total	60	27	33

**Figure 1: genetic patterns of reassortant swine influenza viruses**



*Pathogenicity and transmissibility of novel reassortant and endemic SIVs in pigs*

We have performed 2 pig studies to investigate pathogenesis and transmissibility of selected H3N2 or H1N2 reassortant SIVs compared with the endemic SIVs.

*Experiment #1 (73 pigs):* 3 reassortant H3N2 and 1 endemic H3N2 viruses, which were full-genome sequenced, were selected for in vitro and the pig study: one reassortant H3N2 virus has 5 genes (PB2, PA, NP, M and NS) from the pH1N1; another 2 reassortant H3N2 viruses both contain NP, M and NS genes from the pH1N1, but they have a genetically different NA gene; the endemic H3N2 virus is a triple reassortant virus with genes from avian- (PB2 and PA), human- (HA, NA and PB1) and swine-origin (NP, M and NS) viruses. All 3 novel reassortant H3N2 viruses grew to higher titers than the control endemic H3N2 SIV in canine, swine and human cell lines. In the pig study, all 3 novel reassortant viruses were able to replicate efficiently in lungs and transmit to sentinel animals, similar to the control endemic H3N2 virus. The novel reassortant viruses with 3

genes (NP, M and NS) from pH1N1 were more transmissible when compared to the reassortant virus with 5 genes (PA, PB2, NP, M and NS) from pH1N1. Furthermore, concurrent molecular surveillance showed that the novel H3N2 virus with 3 genes from pH1N1 is continually isolated from Kansas swine herds and becomes a dominant H3N2 virus circulating in swine populations. All these results indicate that novel reassortant H3N2 virus may replace the endemic non-reassortant H3N2 SIV to be the dominant virus circulating in Kansas swine herds. **The results obtained from this study have been presented in the 2012 CRWAD meeting and 2012 ASV conference.**

*Experiment #2 (45 pigs):* 1 reassortant H1N2 (2+6 rH1N2), 1 H1N2 variant isolated from swine (swH1N2v), 1 H1N2 variant isolated from human (huH1N1v) and 1 endemic H1N2 (eH1N2) viruses were selected for the pig study. The selected reassortant H1N2 (2+6 rH1N2) virus has 6 internal genes (PA, PB2, PB1, NP, M and NS) from the pH1N1, and the H1N2 variants isolated from swine (swH1N2v) and human (huH1N1v) have M gene from the pH1N1 and remaining 7 genes from endemic H1N2 viruses. All viruses replicated efficiently in pigs' lungs and successfully transmitted to sentinel animals. However, both variant viruses caused more severe lung lesions in infected pigs when compared to the 2+6 rH1N2 and eH1N2 viruses. Although all four viruses were detected in the lungs of contact animals, the swH1N2v shed more efficiently than the other three viruses in contact animals. Additionally, the huH1N2v displayed delayed and inefficient shedding kinetics in sentinel animals. Taken together, the H1N2 variant viruses are pathogenic and transmissible in pigs and could pose a threat to public and animal health. **The results obtained from this study will be presented in the 2013 ASV meeting.**

## **Discussion**

We have characterized 60 swine influenza viruses which were isolated from diseased or dead pigs from Midwest swine farms with outbreaks of respiratory disease. A total of 27 viruses were identified to be reassortant viruses that contain 1 to 6 genes from 2009 pandemic H1N1 virus (Figure 1): 1 H1N1, 15 H1N2 and 11 H3N2 viruses (Table 1). The pig studies showed that all tested reassortant viruses including H3N2 and H1N2 viruses are pathogenic and transmissible in pigs. The reassortant H3N2 virus containing 3 or 5 genes from

pH1N1 are able to replicate efficiently in pig's lungs and transmit to sentinel animals, similar to the control endemic H3N2 virus. The reassortant H3N2 virus with 5 genes (PA, PB2, NP, M and NS) from pH1N1 was less transmissible when compared to the novel reassortant H3N2 viruses with 3 genes (NP, M and NS) from pH1N1. The result explains why only one H3N2 reassortant viruses with 5 genes from pH1N1 was isolated from swine farms with outbreaks of respiratory disease in our syndromic surveillance program so far. Most of reassortant H1N2 viruses are the H1N2 variant (87%) which was identified from diseased pigs in swine farms with outbreaks of respiratory disease. Pathogenicity and transmission study showed that both human and swine derived H1N2 variant viruses caused more severe lung lesions in infected pigs when compared to the reassortant H1N2 with 6 internal genes from pH1N1 and endemic H1N2 viruses. Furthermore, the swine derived H1N2 variant shed more efficiently than the tested reassortant H1N2, endemic H1N2 and human derived H1N2 viruses in contact animals. All these data indicate that the reassortant H3N2 virus with NP, M and NS genes from pH1N1 and the H1N2 variant will be most likely maintained and become prevail in swine herds, which is in agreement with the syndromic surveillance findings that both viruses are the major novel reassortant viruses found in pigs of Midwest swine farms. They could pose a big threat to the swine industry. In order to control swine influenza, it is first necessary to understand the pathogenicity and transmissibility of these novel viruses and the potential risk of these novel viruses to the swine industry. The results obtained from the funded project have provided insight and knowledge on these novel reassortant viruses for control of this emerging and endemic disease in swine herds and protection of the US swine industry.

The goal of the project 12-095 (Exhibit A) is: a) to sequence and characterize SIVs (15 viruses proposed in the original project, Budget item 3) isolated from diseased pigs in swine farms with outbreaks of respiratory diseases and to perform pig studies required by the specific objectives b) and c). We have over-completed the specific objective a) "60 field isolates including 29 viruses with full-genome sequence and 31 viruses with partial-genome sequence (HA, NA and M genes)" that is over our original budget. We also performed 2 pig studies required by the specific objective b) to investigate pathogenesis and transmissibility of selected novel reassortant H3N2 and H1N2 viruses. In these 2 pig studies 118 pigs (73 pigs in Experiment #1 and 45 pigs in

Experiment #2) were used that are also over our original budget for 101 pigs for the whole project (Budget item 7). Due to the limited budget, we could not conduct another pig study required by the specific objective c) to investigate which genotype virus will be maintained and prevail in swine herds. However, our syndromic surveillance data showed that both reassortant H3N2 virus with NP, M and NS genes from pH1N1 and the H1N2 variant viruses are the major viruses found in pigs of Midwest swine farm, indicating that they will be most likely maintained and become prevail in swine herds. The study on which genotype virus will be maintained and prevail in swine herds will be conducted later if additional funds are available.