RESEARCH REPORT
SWINE HEALTH

Title: Evaluation of two novel live attenuated swine influenza vaccines against newly emerging H3N2 virus infection and transmission – **NPB #14-004**

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Industry Summary:

After the 2009 pandemic, variant H3N2 swine influenza viruses (H3N2v SIV) carrying genes from pandemic H1N1 emerged in pig farms. The objective of this study is to investigate whether the two live vaccine candidates developed in our lab would provide protection to newly emerging H3N2 SIV infection and control virus transmission in pigs.

Two pig trials were conducted for the purpose of the study. First was to evaluate the pathogenicity and transmissibility of H3N2v SIV. A group of pigs (principal infection group) were infected with H3N2v SIV. Then, we introduced a group of healthy pigs (contact group) into the same pen to investigate the direct contact transmission. Contact group stayed with principal infection group for 3 days. The principal pigs infected with H3N2 SIV started to shed large amount of virus in nasal mucus at 24 hours after the infection. The contact pigs also started to shed virus in their nasal mucus at 24 hours after contact with principal group. H3N2v virus infection did not cause fever and other SIV symptoms such as coughing, anorexia, depression but induced lung lesion, which was observed in both principal infection and contact groups. After assessment of pathogenicity and transmissibility of H3N2v SIV, we conducted a similar transmission study in pigs but this time the principal pigs were vaccinated twice with various SIV vaccines; FluSure XP or live SIV vaccines before the H3N2v virus infection. The contact pigs were introduced into the pen of each principal group after the virus infection. All vaccine groups developed antibody response to H3N2v virus but only FluSure XP vaccine group induced protective antibodies. Vaccination of FluSure XP reduced number of pigs shedding virus as well as the amount of shed virus. However, this delayed virus transmission resulted delayed viral clearance in its contact group. Although FluSure XP and live SIV vaccines provided partial protection against A/swine/Kansas/11-110529/2011 (H3N2v) infection since no virus was detected in lung tissues in vaccine groups, both commercial and live vaccines failed to effectively control the transmission of H3N2v virus to healthy contact pigs. Of note that herd vaccination of FluSure XP may control the H3N2v transmission at some level since all the pigs in the herd developed immune responses against H3N2v. In conclusion, inability of vaccines to control the transmission of emerging variant viruses emphasizes the importance of continuous surveillance of SIV circulating in swine farm and vaccine updates.

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Keywords: include at least 5 keywords
Swine influenza virus, live attenuated influenza vaccine, variant H3N2, FluSure XP, influenza virus transmission

Scientific Abstract:

Major subtypes of swine influenza viruses circulating in North America are H1N1, H3N2, and H1N2. Then, SIVs keep evolving and produced variants. H3N2 variant, a newly emerging SIV after the 2009 pandemic, belongs to cluster IV H3N2 and carries genes from 2009 pandemic H1N1. Previously we developed two potential live attenuated vaccines against swine influenza. In this study, we investigated whether our two novel live attenuated swine influenza vaccines would provide protection to newly emerging H3N2 virus infection and to decrease virus transmission in pigs. Avian origin H1N1 SIV was used as a backbone of both live vaccines. R345V has the modification of H1N1 swine influenza virus (SIV) hemagglutinin (HA) cleavage site to render the elastase sensitivity, whereas SIV/606 carries double H1 and H3 (cluster I) subtype of HAs. To evaluate the vaccine efficacy of two live vaccines against infection and transmission of H3N2v, groups of pigs were vaccinated before the virus infection. A commercial SIV vaccine (FluSure XP, Zoetis) was included in this study as a control as it contains H3N2 strain, which belongs to same cluster as H3N2v virus. After the virus infection, groups of contact pigs were housed in the same pen with H3N2v virus infected groups. Although use of live vaccines induced antibody responses to H3N2v, these antibodies lacked hemagglutinin inhibition (HAI) activity while FluSure XP induced antibodies with level of HAI. It implied that there was no or limited protection by live vaccine induced antibodies. H3N2v virus was released in nasal mucus in a day after the infection. In terms of, SIV/606 live vaccine group was comparable to PBS control group. R345V vaccination also failed to control the transmission of H3N2v while vaccination of FluSure XP reduced amount of shedding virus and number of animals shedding the virus. However, once the healthy pigs, which have no protective immunity against H3N2v virus, had contact with infected animals, virus infection occurred and contact pigs started to release the virus. Results of lung lesion score and virus titer in lung and lung lavage fluid suggested that both live vaccines provided partial protection to limit the virus replication in lower respiratory tract.

Introduction: An overview of the researchable question and its importance to producers.

Influenza A virus is a highly infectious respiratory pathogen of mammals. Influenza virus infection in swine may cause significant morbidity, resulting in a substantial economic loss in the farm. In addition to the direct economic loss, influenza virus infection in pigs poses public health concerns as we have seen during 2009 pandemic. Vaccination is the primary measure of controlling swine influenza virus (SIV) infection. Although inactivated swine influenza vaccines are available, live attenuated vaccines provide immunity superior to that induced by conventional inactivated vaccines, and thus provide better, broader and more profound protection. Especially, live attenuated vaccines induce mucosal immunity, which reduces the amount of virus shedding to the environment, thus will significantly reduce the virus transmission. Cold adapted live attenuated vaccines have been developed for human and equine influenza viruses. However no live attenuated vaccines are available for swine influenza virus. In the last couple of years, we developed two live attenuated vaccine candidates for swine influenza. The uniqueness of our vaccine candidates is that the replication of our attenuated viruses highly depends on the supplement of either elastase or neuraminidase *in vitro*, thus they only replicate one cycle in vivo. One cycle replication of the vaccine viruses enables the stimulation of the immune system without causing disease. Thus our attenuated vaccines are safer in the way that it significantly reduces the chance of getting recombination with wild type virus.

The success of the proposed study will greatly contribute to the arsenal of tools to prevent SIV infection and reduce SIV transmission in pigs. This will allow us not only to control the circulating strains but also to quickly respond to any new emerging SIV strains. This would be of significant economic benefits to the swine industry and to our society as well.

The proposal will examine the ability of novel SIV vaccines developed by reverse genetics to decrease viral shedding and transmission and to provide broad protection against multiple strains. This is one of the research priorities under “SWINE HEALTH-General Swine Disease” set by the NPB.

With the support from National Pork Board during 2007-2008 and Saskatchewan Agriculture Development fund during 2008-2011, we developed two potential live attenuated vaccines against swine influenza. R345V is based on the modification of swine influenza virus (SIV) hemagglutinin (HA) cleavage site, whereas SIV/606 carries double H1 and H3 subtype of HAs. Our previous studies have demonstrated both vaccine candidates are highly attenuated in pigs (do not cause disease), however they could induce robust

immune responses and provide superb protection against several SIV strains infection, including pandemic H1N1 virus.

Objectives:

The objective of this proposal is to investigate whether our two novel live attenuated swine influenza vaccines would provide protection to newly emerging H3N2 virus infection and to decrease virus transmission in pigs.

Our study focused on the following specific aims:

1) Analysis of immune responses after vaccination and virus challenge in pigs.
2) Evaluation of protection efficacy and transmission capacity after vaccination and virus challenge in pigs.

Materials & Methods:

1. Live attenuated influenza vaccines and variant H3N2 virus

Two live attenuated influenza vaccines (LAIV), R345V and SIV/606, were propagated and in MDCK cells, and purified using sucrose density gradient ultracentrifugation, titrated, and stored at in -80°C until use. Both LAIVs were originated from the fully avian SIV isolate A/swine/Saskatchewan/18789/02 (H1N1). R345V has V instead R at position 345 of hemagglutinin and this mutation renders the virus elastase sensitive instead trypsin. SIV/606 is carrying recombinant NA segment, of which NA ectodomain is replaced with H3 HA ectodomain. SIV/606 requires neuraminidase along with trypsin for efficient virus replication.

Influenza A/swine/Kansas/11-110529/2011 (H3N2v) was kindly provided by Dr. Wenjun Ma (Department of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, USA). H3N2v virus carries 3 genes (NP, M, and NS) from 2009 pandemic H1N1 virus, and the rest of the genes from triple reassortant H3N2 virus.

2. Pathogenicity and transmissibility of H3N2v virus

In vivo study was conducted at Vaccine and Infectious Disease Organization-International Vaccine Centre, University of Saskatchewan in accordance with the ethical guidelines of the University of Saskatchewan and Canadian Council of Animal Care. The first *in vivo* study aimed to evaluate the transmissibility and pathogenicity of H3N2v virus in pigs. The study was performed as shown in table 1. For the study, thirteen of 6-week-old pigs were purchased from a farrow to finish farm, located in Saskatchewan, Canada. This farm had no history of SIV and PRRSV outbreaks and did not use SIV vaccine. Pigs were sero-negative to SIV tested by commercial SIV ELISA kits (Idexx Inc., USA) and by antigen specific ELISA developed in house. Pigs were divided into three groups and housed in separate rooms. Pigs in principal infection group were intranasally inoculated with 4×10^6 PFU of A/swine/Kansas/11-110529/2011 (H3N2v) in 1ml of MEM. Pigs in control group were given 1ml of MEM. Pigs in contact group were introduced into the pen with principal infection group on 2 days post infection (dpi). Pigs in control group were kept in a separate room until the end of the experiment. Clinical symptoms including body temperature was monitored and nasal swabs were collected once a day from all pigs. Pigs in infection group were euthanized on 5 dpi. Contact and control pigs were euthanized on 7 days post contact (dpc). At necropsy, SIV infection induced gross lesions in lungs were recorded by a veterinarian. Bronchoalveolar lavage fluid (BALF) and lung biopsies were collected at necropsy.

Table 1 A/swine/Kansas/11-110529/2011 (H3N2) virus transmission study

Group (*number* of pigs)	Day									
	0	1	2	3	4	5	6	7	8	9
Principal infection (*n*=5)	VI*					N***				
Contact (*n*=5)			C**	C**	C**					N***
Control (*n*=3)										N***

*VI : Virus infection
**C : Contact with principal infection group
***N : Necropsy

3. Vaccine study design

The second study was designed to evaluate the vaccine efficacy of two live vaccines against infection and transmission of A/swine/Kansas/11-110529/2011 (H3N2v). A commercial killed SIV vaccine (FluSure XP, Zoetis) was included in this study as a control. According to proprietary information, FluSure XP is a commercial killed vaccine that contained cluster IV H3N2 subtype.

For the study, forty three of 3-week-old pigs were purchased from the same swine farm. Before any vaccination, pigs were screened by ELISA as described earlier. Pigs were randomly allocated into 9 groups. Group assignment and study schedule were summarized in table 2 and Fig. 1. After one week of acclimatization (day 0), the pigs in group A to D were vaccinated with PBS, FluSure XP, and two of LAIVs. PBS (2ml) and FluSure XP (2ml) were vaccinated intramuscularly. LAIVs, R345V and SIV/606 (4×10^6 PFU in 1ml MEM) were vaccinated intranasally. The pigs in group A to D received boost vaccines on day 21 and then were intranasally inoculated with 4×10^6 PFU of A/swine/Kansas/11-110529/2011 (H3N2v) in 1ml of MEM on day 37. The pigs in group E to H were introduced into the same pen with the pigs of principal infection groups on 2 dpi (day 39). The pigs in group I housed in a separate room throughout the experiment. We monitored clinical signs and body temperature, and collected nasal swab daily. The pigs in group A to D were euthanized at 5 dpi (day 42). The pigs in contact groups (E to H) and control group (I) were euthanized at 7 dpc (day 46). Blood samples were collected on day 0, 21, 37 from the pigs in groups A to D and on day 0, 39, 46 from the pigs in groups E to I. SIV affected gross lesions in lungs were examined and BALF and lung tissues were collected at necropsy.

Table 2 Group assignment and description of vaccination and virus infection

Group (*number* of pigs)	Group description	Virus challenge
A (*n*=5)	Vaccine control (PBS)	Yes
B (*n*=5)	FluSure XP	Yes
C (*n*=5)	R345V LAIV	Yes
D (*n*=5)	SIV/606 LAIV	Yes
E (*n*=5)	Contact of group A	No
F (*n*=5)	Contact of group B	No
G (*n*=5)	Contact of group C	No
H (*n*=5)	Contact of group D	No
I (*n*=3)	No treatment control	No

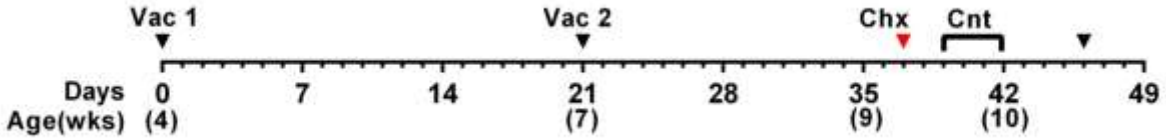

Figure 1. Vaccination, virus challenge and transmission study schedule.

Results:

1. Pathogenicity of A/swine/Kansas/11-110529/2011 (H3N2v) virus.

H3N2v virus infection did not cause apparent clinical symptoms related to influenza infection including fever in both principal group and contact group (Fig. 2). Lung lesions induced by influenza infection was examined by a veterinarian at necropsy. A/swine/Kansas/11-110529/2011 (H3N2v) virus infection induced mild to moderate lung lesions in principal group and contact group. The score ranged from 5 to 10 (Fig. 3).

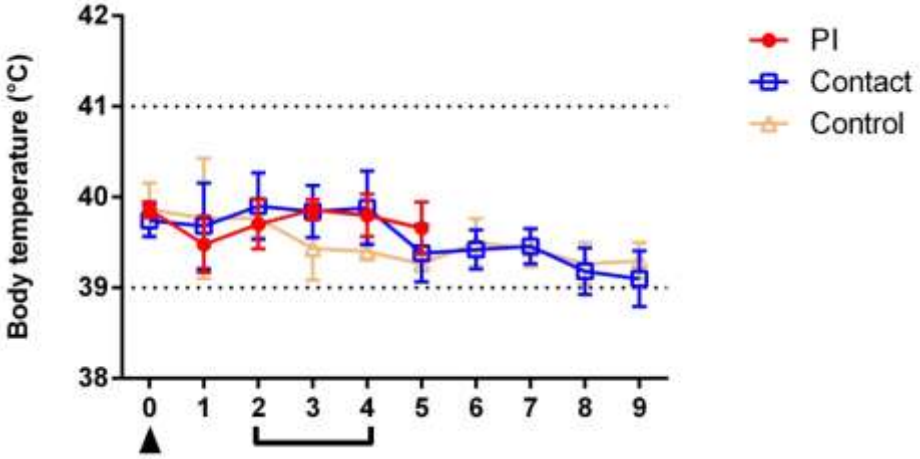

Figure 2. Body temperature was measured daily. Arrow head indicates virus challenge and contact period was marked. Body temperature range considered normal was indicated with dotted line.

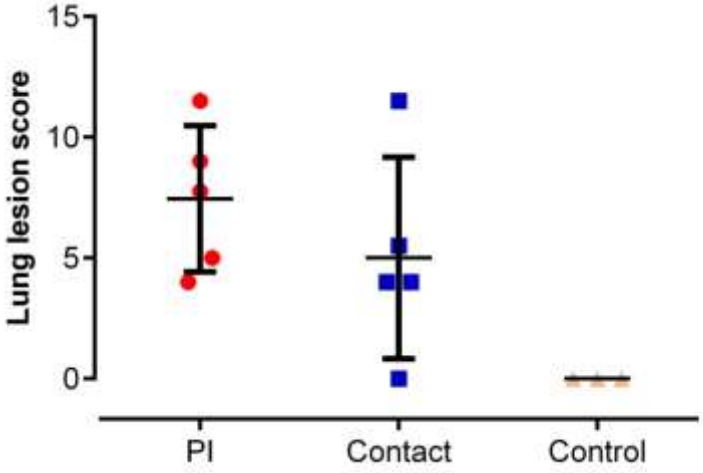

Figure 3. Lung lesion score.

Virus released in lower respiratory tract was measured by titration using lung lavage collected at necropsy. Virus was detected in lung lavage collected from principal pigs but not in the ones from contact pigs (Fig. 4). Although no virus was isolated in lung lavage, presence of virus in lower respiratory tract of contact pigs was confirmed by the isolation of virus in lung tissue homogenates (Fig. 5)

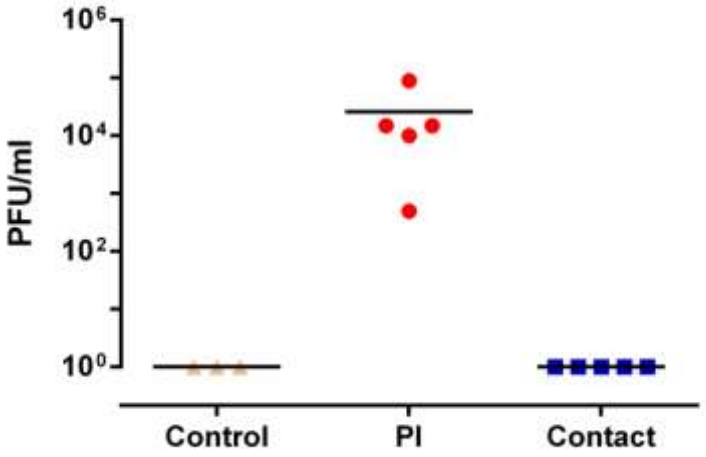

Figure 4. Virus detection in lung lavage samples.

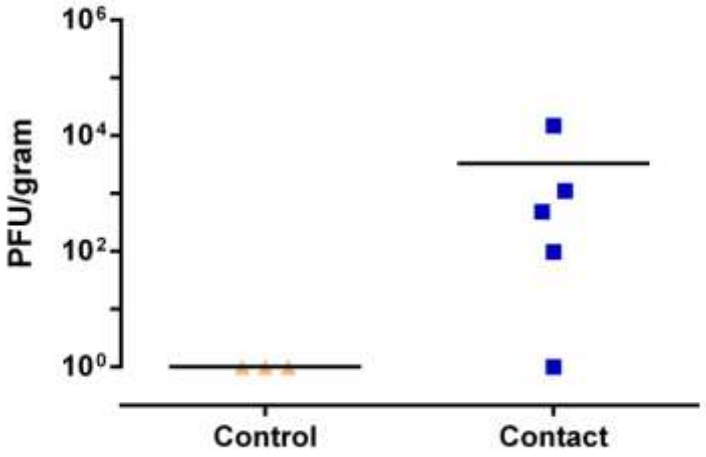

Figure 5. Virus replication in lung.

2. Transmissibility of A/swine/Kansas/11-110529/2011 (H3N2v) virus.

Nasal swabs were collected daily to measure the shedding of A/swine/Kansas/11-110529/2011 (H3N2v) virus (Fig. 6). Principal pigs started to shed virus in nasal cavity on 1dpi and virus titer sustained until euthanasia. The titer of virus was similar in all the five pigs in principal infection group during the observation period. Contact pigs initiated virus shedding on 1 day post contact (dpc) with principal pigs although the virus titers varied. The shedding virus titer in contact group was comparable to that of principal infection group on 2 dpc (day 4). All contact pigs shed similar amount of virus by 2 dpc and maintained viral titer until 6 dpc. At necropsy (7 dpc), decreased viral titer was noted in the contact group. Four out of five nasal swab samples of contact group on 7 dpi had no virus.

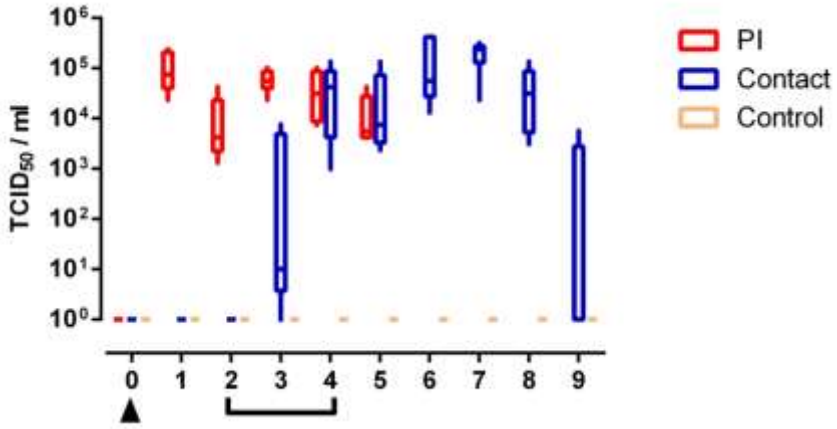

Figure 6. Transmission dynamics of A/swine/Kansas/11-110529/2011 (H3N2v). Arrow head indicates virus challenge and contact period was marked.

3. Vaccine induced A/swine/Kansas/11-110529/2011 (H3N2v) virus specific antibody responses.

Serum IgG specific to challenge virus was evaluated by ELISA after the vaccination (Fig. 7). Serum IgG titer of pigs in all 9 groups was under 100 on day 0. Four groups of pigs (*n=5*) were vaccinated with either PBS, commercial vaccine, or two LAIVs on day 0. Serum IgG titers of four vaccine groups, including PBS group, were increased on day 21. Serum IgG titers of vaccine groups, except PBS group, were increased after the boost vaccination as shown on day 37.

Although increased serum IgG titers in all three vaccine groups, only group B had hemagglutination inhibition activity (table 3). All pigs vaccinated with commercial killed vaccine developed HAI titer over 160 while the pigs in two LAIV groups were all under 20 of HAI.

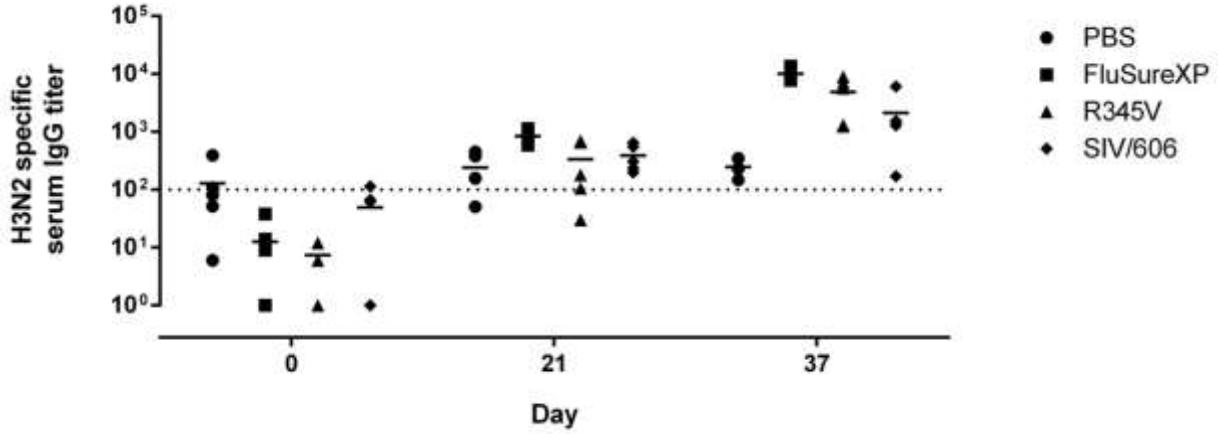

Figure. 7. A/swine/Kansas/11-110529/2011 (H3N2) specific IgG titers in serum collected before and after the vaccination. Bar is mean value of each group.

Table 3 Hemmagglutinin inhibition titer of individual pig on day 37

Group (*n*=5)	HAI titer				
PBS	<20	<20	<20	<20	<20
FluSure XP	320	1280	160	160	320
R345	<20	<20	<20	<20	<20
SIV/606	<20	<20	<20	<20	<20

4. Vaccination and A/swine/Kansas/11-110529/2011 (H3N2v) virus transmission

Nasal shedding of each group and number of individual were shown in Fig. 8 and table 4. Challenge virus was detected on 1 dpi in nasal swabs in some pigs in group A and B. All five pigs in group A, vaccinated with PBS, had nasal virus shedding on 1 dpi (geometric mean of $10^{3.17}$). Five pigs in groups D, vaccinated with SIV/606, shed viruses comparable to group A (geometric mean of $10^{3.12}$). Two pigs in group B, vaccinated with FluSure XP, and 3 pigs in group C, vaccinated with R345V, shed virus on 1 dpi. Over the observation period, only one pig in group B consistently shed virus. In group C, all five pigs shed on 2 dpi (median titer). By 4 dpi, virus shedding started to decline in two LAIV groups. By the time we euthanized animals (5 dpi), 2 pigs in group C and one pig in group D shed virus and none of pig in group B shed virus.

The contact groups were introduced into the same pen with infection groups in 2 dpi and shared the pen with infection groups for three days until the pigs in infection groups were euthanized on 5 dpi. The contact group with group D, vaccinated with SIV/606, started nasal shedding on 1 dpc while the rest contact groups did not shed virus until 2 dpc. On 2 dpc, all four contact groups had pigs that shed virus. All five pigs in group E, contact pigs for PBS vaccine group, shed virus while 2, 4, and 3 pigs shed in group F, G, H, respectively. More numbers of pigs shed virus in each group and all pigs in all contact groups shed virus in nasal mucosa by 4 dpc. Contact pigs in groups E, G, and H ceased viral shedding on 6 dpc and none of animal in these groups shed virus on 7 dpc except the pigs in group F. Two of the contact pigs in group F shed virus on 7 dpc.

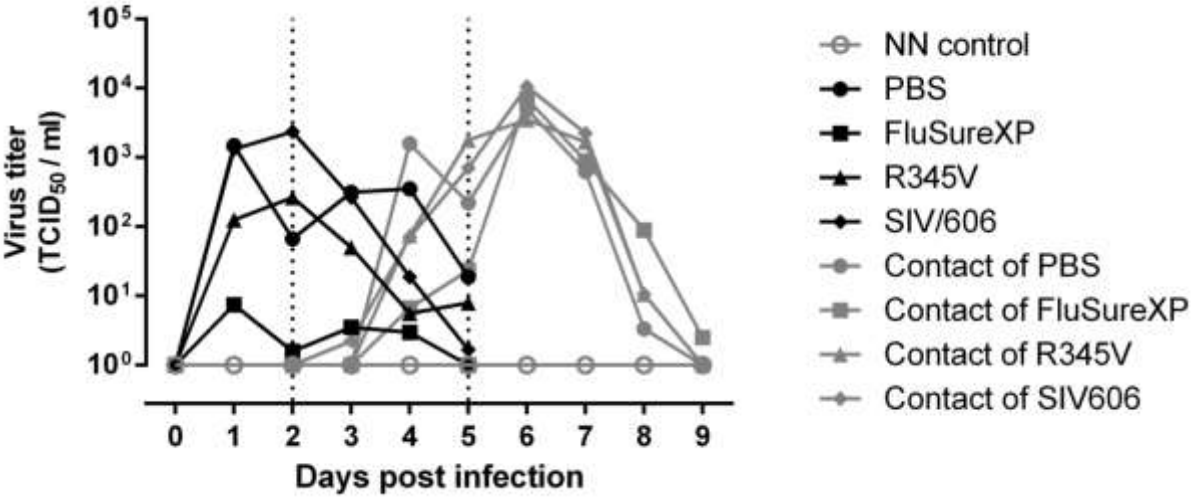

Figure. 8. Transmission dynamics of A/swine/Kansas/11-110529/2011 (H3N2) after the vaccination. Each point represents geometric mean of virus titer.

Table 4 Number of pigs shedding virus

Group (*n*=5)		Days post infection									
		0	1	2	3	4	5	6	7	8	9
A	PBS	0	5	4	5	5	3				
B	FluSure XP	0	2	1	1	1	0				
C	R345V	0	3	5	4	2	2				
D	SIV/606	0	5	5	5	2	1				
E	Contact of A			0	0	5	4	5	5	2	0
F	Contact of B			0	0	2	3	5	5	5	2
G	Contact of C			0	0	4	5	5	5	3	0
H	Contact of D			0	2	3	5	5	5	4	0

5. Vaccination and A/swine/Kansas/11-110529/2011 (H3N2v) virus induced pathology

Lung lesion was examined and scored as for the criteria of the protective efficacy of vaccines against A/swine/Kansas/11-110529/2011 (H3N2v) (Fig. 9). Three pigs in group I (non-vaccinated, non-challenged group) had not developed pathological changes in lungs. Four out of 5 PBS vaccinated pigs (group A) also free of lung lesion. One animal in this group developed lung lesion with the score of 7.5. Among the vaccine groups, FluSure XP vaccinated pigs (group B) developed the least lung lesion. Four pigs in this group had no SIV lesion and one animal had mild lesion (score of 1.5). Pigs belong to two LAIV vaccinated groups (group C and D), developed lung lesions with score ranging from 5.5-10.9 and 2.5-6.25, respectively. All pigs in contact groups developed lung lesions with score ranging from 0.15 to 25. Only one animal in group G had no lesion.

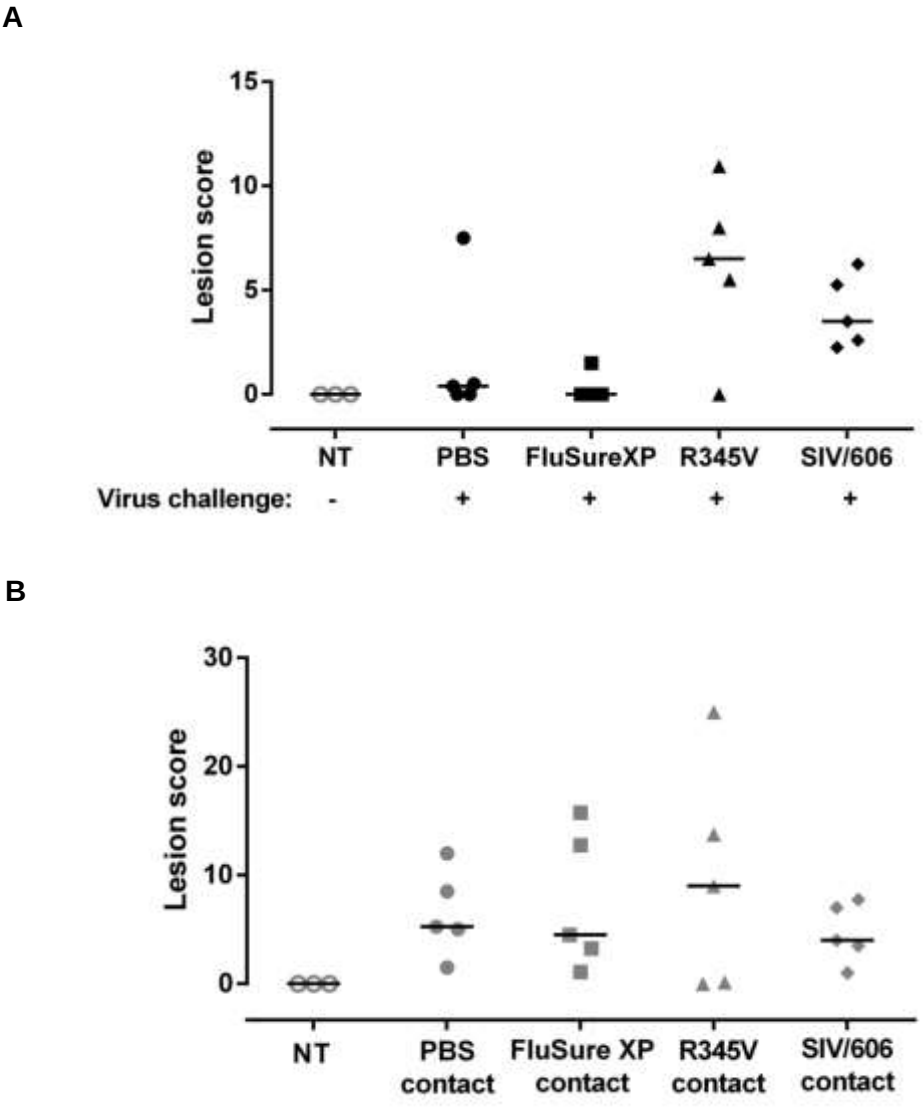

Figure 9. Lung lesion score of vaccinated and challenged groups (A) and contact groups (B). Dots are individual pigs and the bar represents median value of the group.

Virus replication in lower respiratory tract was measured using BALF and lung tissues (Fig. 10 and 11). We did not detect any virus in lung tissues and BALF of three pigs in group I. All PBS vaccinated pigs in group A had virus in their BALF (geometric mean titer of $10^{2.46}$) and four of them had virus in lung tissue (geometric mean titer of $10^{2.44}$). Pigs in FluSure XP vaccinated group (group B) was free of virus in BALF while some pigs in two LAIV vaccinated groups, C and D, had virus in their BALF. Three pigs in group C and two in group D had virus in BALF (geometric mean titer of $10^{0.6}$ and $10^{0.77}$, respectively).

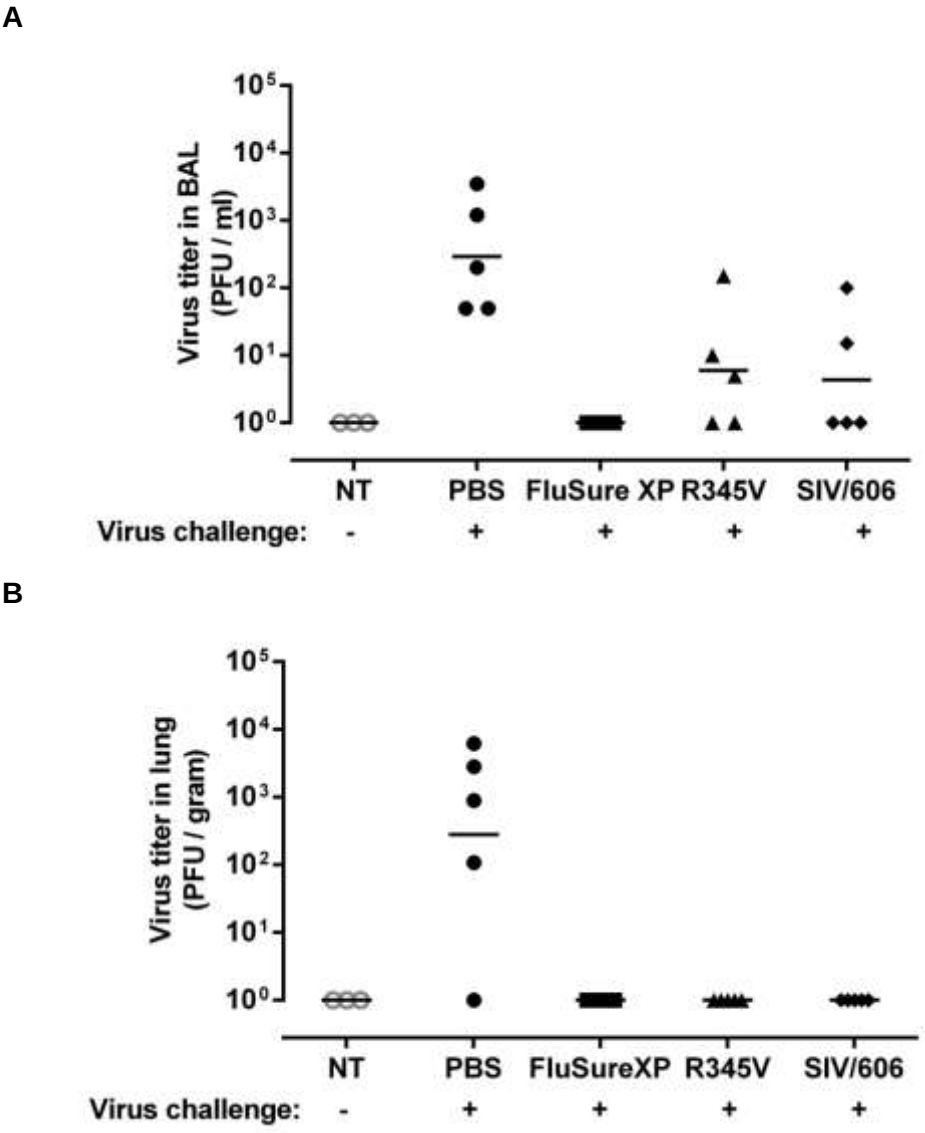

Figure 10. Virus titer in BALF (A) and lung tissues (B) of vaccinated and challenged groups (A-D).

BALF and lung tissues of contact groups were free of virus except the contact group F, contact group of FluSure XP vaccinated group B (Fig. 11). Three of pigs in this group had virus in both BALF and lung tissues (geometric mean titer of $10^{1.77}$ and $10^{1.92}$, respectively).

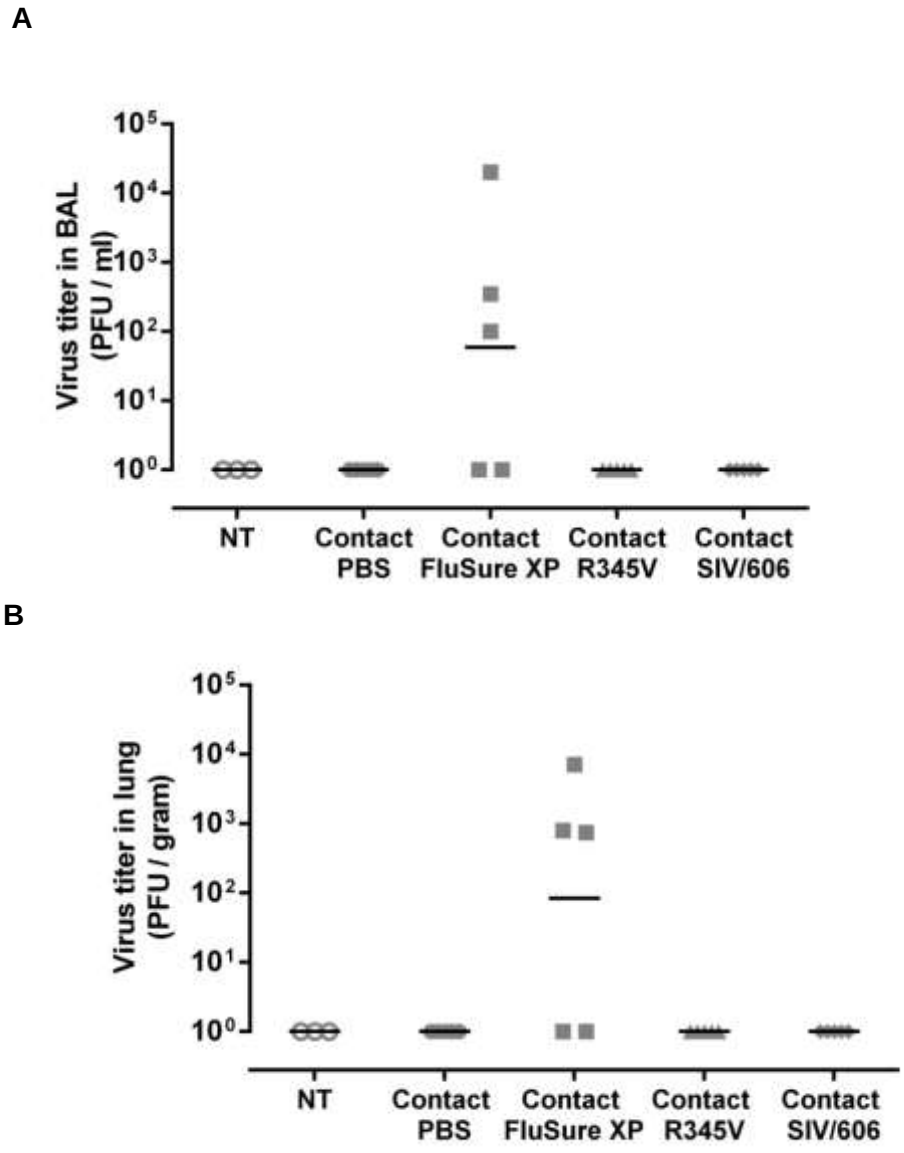

Figure. 11. Virus titer in BALF (A) and lung tissues (B) of contact groups (E-H).

Discussion:

After the outbreak of pandemic H1N1, reassortant H3N2 SIVs carrying genes from the pandemic H1N1 have been detected in North American swine population. These H3N2 SIVs, called H3N2 variants (H3N2v), transmitted back and forth between human and pigs. The numbers of human cases that have been continuously increased in past years implies the possibility of another influenza pandemic. Since, H3N2v is one of major SIV genotypes that circulating in the pigs that including this genotype in the vaccine formula would confer the better protection coverage in the farm. In this regard, we have evaluated two LAIV candidates against A/swine/Kansas/11-110529/2011 (H3N2v). Our ELISA and HAI results suggest that LAIV vaccination induced some cross reactive antibody responses but these antibodies may not have neutralizing activity to A/swine/Kansas/11-110529/2011 (H3N2v). Commercial vaccine, FluSure XP, which was known to contain cluster IV H3N2 subtype, induced HAI titer. Such result coincided lung lesion score after the viral challenge of A/swine/Kansas/11-110529/2011. FluSure XP vaccinated group was free of gross lung pathology while other LAIV groups developed lung lesions. In terms of blocking viral transmission, FluSure XP vaccination reduced number of pigs shedding virus as well as the titer of shedding virus. However, this delayed virus transmission resulted delayed viral clearance in its contact group. Overall, vaccination of FluSure XP as well as our two LAIVs was not able to control the transmission of A/swine/Kansas/11-110529/2011 (H3N2v). However, our result suggested that herd vaccination of FluSure XP may control the H3N2v transmission at some level since all the pigs in the herd developed immune responses against H3N2v. Although these LAIV vaccines did not prevent viral transmission, vaccinated groups provided some level of protection against A/swine/Kansas/11-110529/2011 (H3N2v) infection as shown in viral titer in lungs.