RESEARCH REPORT
SWINE HEALTH

Title: Evaluation of genetic diversity and dynamics of virus infection in a wean-to-finish pig population. Identification **- NPB # 12-068**

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Industry Summary:

Influenza A virus (IAV) can persist in populations for prolonged periods of time. The objectives of this study were to estimate the diversity of IAV in an infected growing pig population, and to determine the extent of pig re-infection with resident viruses. This study is important in order to understand why IAV persist in swine populations. One hundred thirty two pigs were selected at weaning and tested to determine their weekly flu status, and monthly to evaluate seroconversion. Pigs that tested positive for the presence of virus were selected and samples were submitted for whole genome sequencing using next generation sequencing techniques. Sequencing was conducted directly from nasal swabs to capture the breath of genetic diversity.

Our results indicated that IAV spread rapidly after weaning and that all pigs became infected despite the presence of immunity at weaning. Interestingly there were three genetically distinct viruses identified in the population that caused two distinct IAV epidemics. The first and second epidemic peaks were dominated by H1 and H3 viruses respectively. However both subtypes were identified co-circulating during both epidemic peaks. This is interesting since it shows that multiple IAV strains and subtypes can co-circulate within a population at different levels. We also showed that a significant percentage of pigs became re-infected with IAV and most often this re-infection was with a different IAV subtype. However, there were some pigs that became re-infected with the same subtype but a different strain, and other pigs that became re-infected with the same strain.

Our results provide the first basic understanding on influenza virus diversity and transmission in pigs after weaning in a commercial herd. We identified conditions for IAV persistence and reassortment after weaning. This information is relevant in order to understand why influenza persists in populations and what risk endemically infected populations represent in the generation of new viruses. Our results should assist in the development of better vaccines and strategies to control and reduce the impact of IAV in pigs.

Keywords: Influenza A virus (IAV), IAV diversity, infection, transmission.

Scientific Abstract:

Influenza A virus (IAV) can persist in populations for prolonged periods of time. The objectives of this study were to estimate the diversity of IAV in an infected growing pig population, and to determine the extent of which pig re-infection with resident viruses occurs.

A cohort of 132 out of 2200 commercial pigs were randomly selected at weaning and followed for 15 weeks in a wean-to-finish herd (WF). Pigs were individually identified and nasal swabs were collected weekly to test them by IAV real time RT-PCR. Serum samples were collected monthly to evaluate seroconversion by ELISA. A subset of 96 samples from pigs that tested positive more than once were selected for complete genome sequencing and subtyping using next generation sequencing technologies (NGS). Phylogenetic analysis was performed by gene segment to estimate the genetic relationship between viruses over time. The proportion of positive and negative samples was compared between weeks, and the weekly and period prevalence were estimated. Finally, the proportion of pig re-infection defined as two or more positive RT-PCR results in non-consecutive weeks,was estimated and the phylogenetic relationship of viruses within pigs evaluated.

Ninety nine percent of the pigs tested IAV positive at least once during the study, and 100% seroconverted by week 12. Based on sequencing there were three genetically distinct viruses, two H1 viruses and one H3. While the first IAV epidemic (week 2) was dominated by H1 viruses, the second one (week 7) was dominated by H3 viruses. However, using deep genome sequencing we were able to identify H1 viruses in all weeks and H3 in most of the weeks. Eighty three percent of the pigs became re-infected with IAV, with most of them becoming re-infected with a different subtype of IAV. However a few pigs were re-infected with IAV of the same subtype and some with IAV of the same genetic cluster.

Our study indicated that IAV was widespread, transmitted rapidly in pigs after weaning and that IAV genetic diversity was significant. Deep genome sequencing was helpful to determine co-infections in pigs and that viral populations change over time with the distribution of subtypes changing as well. Re-infection was common and it was more likely to happen with antigenically distinct viruses of a different subtype.

Our results provide a basic understanding on influenza virus diversity and transmission in pigs after weaning in a commercial herd. We identified conditions for IAV persistence and reassortment after weaning. This information is relevant in order to understand why IAV persists in populations and what risk endemically infected populations represent in the generation of new viruses. Our results should assist in the development of better vaccines and strategies to control and reduce the impact of IAV in pigs.

Introduction:

Influenza A virus (IAV) is an important swine respiratory pathogen that causes reduced herd performance due to high morbidity, low mortality and increased susceptibility to secondary infections (Olsen et al., 2006). The direct cost of flu to producers has not been quantified but values as high as $10/pig have been reported for infected herds (Donovan, 2008). Influenza virus represents a constant

threat to both animal and public health as influenza viruses are RNA viruses with ability to change and reassort, resulting in new variants with a new host range. Influenza viruses are shared by many host species. Humans, birds and/or pigs can become infected with viruses originating from any of these species (Olsen et al., 2002; Newman et al., 2008; Howden et al., 2009).

The epidemiology of IAV transmission within and between species is extremely complex and not completely understood (5, 7). The pig has been blamed to be the mixing vessel for IAVs (8) from different species but limited information is available on swine IAV evolution under field conditions. IAV can persist for prolonged periods of time in swine populations, and swine herds can test positive for one or multiple IAV subtypes(9). Homologous vaccination to IAV can prevent infection with IAV in pigs, while heterologous vaccination can reduce transmission between pigs(10, 11). However the mechanisms (viral or host dependent) that influence IAV diversity, evolution and maintenance in swine populations are not clear. Therefore the overall objective of this research project was to further characterize the transmission and evolution of IAV in pigs after weaning in a commercial swine farm characteristic of US swine rearing conditions.

Objectives:

a) Determine virus diversity and virus change over time in an endemically infected growing pig population.

b) Determine the extent of re-infection in endemically infected groups of pigs, with resident viruses.

Materials & Methods:

A commercial wean to finish herd (WF) was conveniently selected for this study. The herd was single sourced and both the sow herd and WF were previously diagnosed positive to IAV by RT-PCR. At arrival, 132 out of 2200 three week-old pigs were randomly selected and ear tagged. Sample size was calculated at a 95% confidence level to detect at least one IAV positive pig if the prevalence was 2.5% or higher.

Individual nasal swabs were collected at arrival and on a weekly basis for 15 weeks. Each swab was tested for IAV by RT-PCR targeting the M gene (12). Two different cutoff values for the RT-PCR cycle threshold (CT) were used to consider a sample positive to IAV (<35 or 36-40). Weekly and total prevalence were estimated. The number of positive samples was compared between weeks using a chi square test. A set of positive pigs that tested positive, was conveniently selected to amplify the complete genome of IAV using previously described methods (13). Complete genome amplicons were submitted for deep genome sequencing (Ilummina MiSeq) to the BioMedical Genomic Center (BMGC) at the University of Minnesota. Illumina reads were mapped to a reference IAV genome using Bowtie2 (14) and a consensus sequence for each sample was obtained using SAMtools (15). Complete and partial gene sequences were used to determine the subtype of IAV for each sample. Only complete gene sequences were used to estimate the phylogenetic relationship of AV gene segments, and internal gene sequences were used to estimate reassortment events between viruses.

Serum samples were collected from all pigs at arrival and every 4 weeks to determine IAV exposure. Serum samples were tested for antibodies to IAV by ELISA. Samples were considered positive (SN<0.6), suspect (0.6<S/N<0.9) and negative (>0.9 S/N) according to the sample to negative (S/N) ratio (16). The sample to negative ratios (SN) from the ELISA test were compared between weeks using a paired Wilcoxon signed rank test, and considered significant at 0.05. Finally, PCR results and

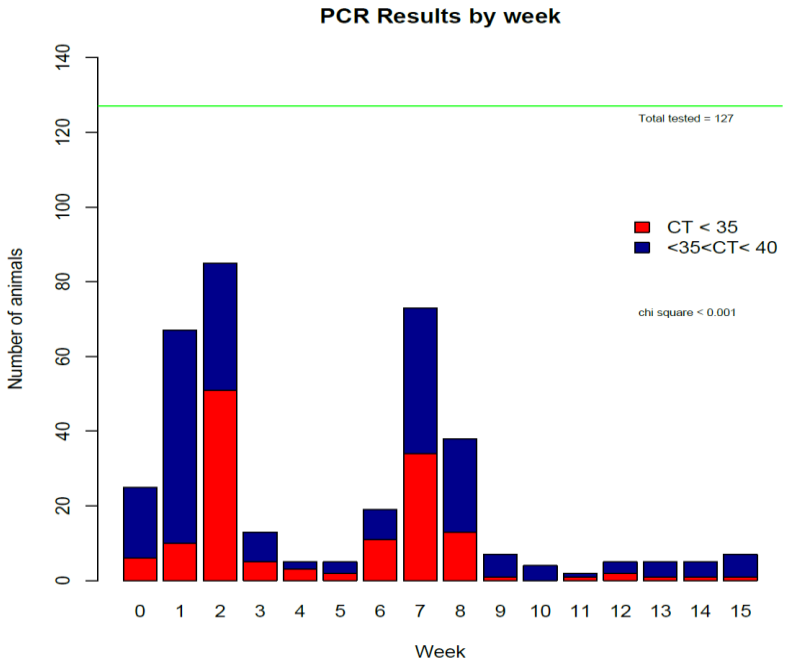
sequences from pigs that tested positive in more than one week were compared to assess the risk of re-infection with IAV over time. Re-infection was defined as two or more positive samples in non-consecutive weeks. This study followed approved University of Minnesota IACUC procedures with protocol number 1207B17281.

Results: Report your research results by objective.

1. *Objective 1: Determine IAV diversity and virus change over time in an endemically infected growing pig population:*

A total of 2032 nasal swabs and 508 serum samples were collected from 127 out of 132 pigs (males=58%, females= 42%) selected at the beginning of the study. Five pigs (3.8%) died during the study and were excluded from the analysis. Seven percent (n=142) of nasal swabs tested positive by RT-PCR (Ct<35) or 18% (n=365) were considered positive if a Ct<40 was used. The number of weekly positive pigs ranged between 2 (week 11) and 85 (week 2) hence the weekly prevalence of IAV in this study ranged between 1.6% and 66.9%. One hundred and twenty six pigs tested positive at least once during the duration of the study for a total prevalence of 99.2% (Ct<40) or 72% (n=95) if a more conservative cutoff value of CT<35 was used. The proportion of positive pigs between weeks was found statistically different ($p<0.05$) and two IAV epidemic peaks were identified, one at week 2 (66.9% positives) and another one at week 7 (57% positives) (Figure 1).

Figure 1. Number of IAV positive pigs by week and RT-PCR cycle threshold value.



Sequencing was performed directly from 96 swab samples. We obtained 680 complete and 210 partial sequences across the different IAV gene segments (Table 1). Two IAV subtypes, H1N1 and H3N2, were identified during the study period. While H1 viruses were found in all weeks, H3 viruses could not be found on weeks 4, 9, 11, 13 and 14 (Table 2). The first and second epidemic peaks were dominated by H1 and H3 viruses respectively (Table 2). However the identification of partial sequences (13 H3 on week 2 and 15 H1 on week 7) indicated the co-circulation of both subtypes during both IAV epidemic peaks. The overall percentage identity of complete HA sequences ranged between 51.2% and 100% with a mean of 76.5%. Three different HA clusters were identified with two distinct H1 and one H3 clusters (Figure 2), and the mean percentage identity within and between clusters is shown in Table 3.

Table 1.Number of IAV sequences by gene segment obtained during the study period.

	Seg1	Seg2	Seg3	Seg4H1	Seg4H3	Seg5	Seg6N1	Seg6N2	Seg7	Seg8	Total
Complete	89	81	88	56	32	79	60	39	93	63	680
Partial	7	14	8	33	34	17	27	34	3	33	210
Total	96	95	96	89	66	96	87	73	96	96	890

Table 2. Number of samples with H1 and/or H2 sequences by week.

Week	0	1	2	3	4	5	6	7	8	9	11	12	13	14	15	Total
No. of samples	5	9	30	2	2	1	9	21	10	1	1	2	1	1	1	96
H1 positive	5	9	30	2	2	1	9	18	6	1	1	2	1	1	1	89
H3 Positive	4	7	14	1	0	1	4	21	10	0	0	2	0	0	1	65

Figure 2. Phylogenetic relationship of complete HA sequences obtained during the study period. a) H1 clusters 1 (dark blue), and 2 (light blue). b) H3 cluster 3 (green)

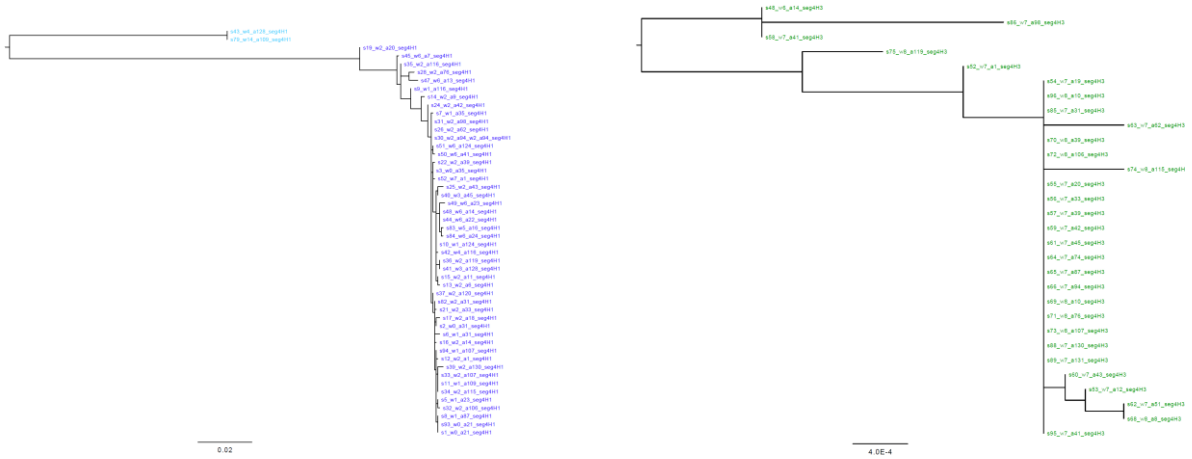
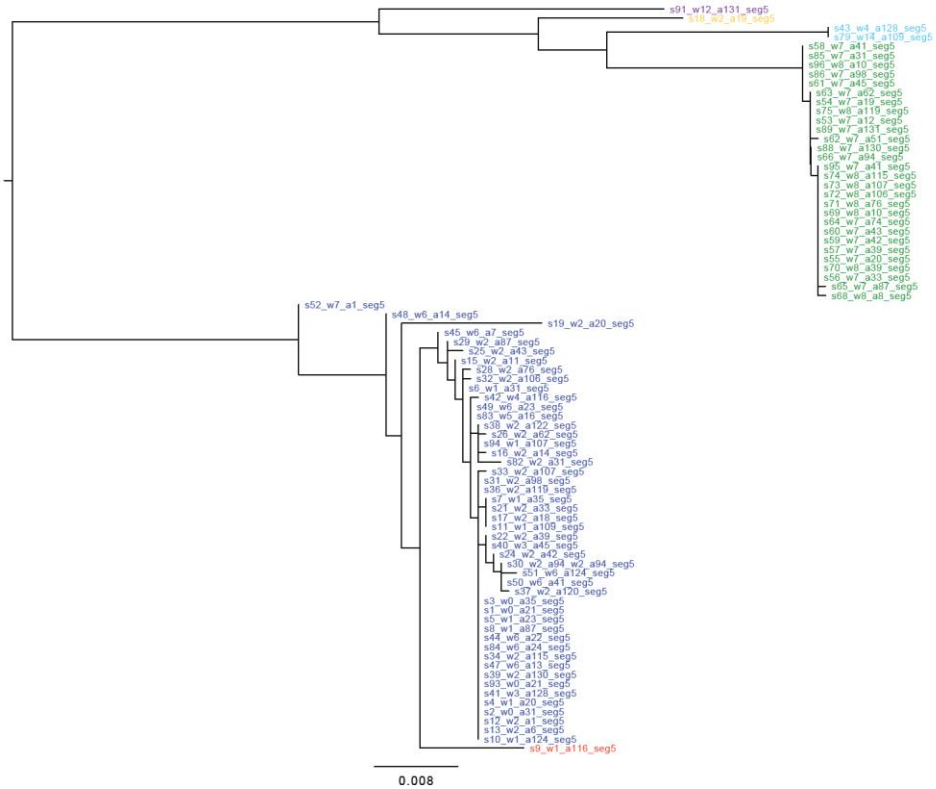


Table 3. Mean percentage identity within and between hemagglutinin gene clusters. A cluster was defined as a group of sequences with 98% identity or higher. While cluster 1 and 2 represent H1 viruses cluster 3 represents H3 viruses. * Number of sequences within cluster. ** Range of the percentage identity between compared clusters.

	Cluster 1	Cluster 2	Cluster 3
Cluster 1 (n=48)*	99.4 (97.1 - 100)**		
Cluster 2 (n=2)	90.8 (90.7 - 92.9)	99.2	
Cluster 3 (n=30)	51.6 (51.2 -52.0)	51.4 (51.2 - 51.5)	99.9 (99.4 - 100)

All other gene segments but segment 5 (Nucleoprotein) and 7 (Matrix) clustered in 3 genetic groups using the same cutoff value used for HA (98% or higher). While 6 different clusters were identified for segment 5 (Figure 3) 13 were identified for segment 8 (not shown).

Figure 3. Phylogenetic relationships of complete nucleoprotein (NP) sequences obtained during the study period. Clusters are color coded 1 (dark blue), 2 (light blue), 3 (green), 4 (purple), 5 (orange) and 6 (red).

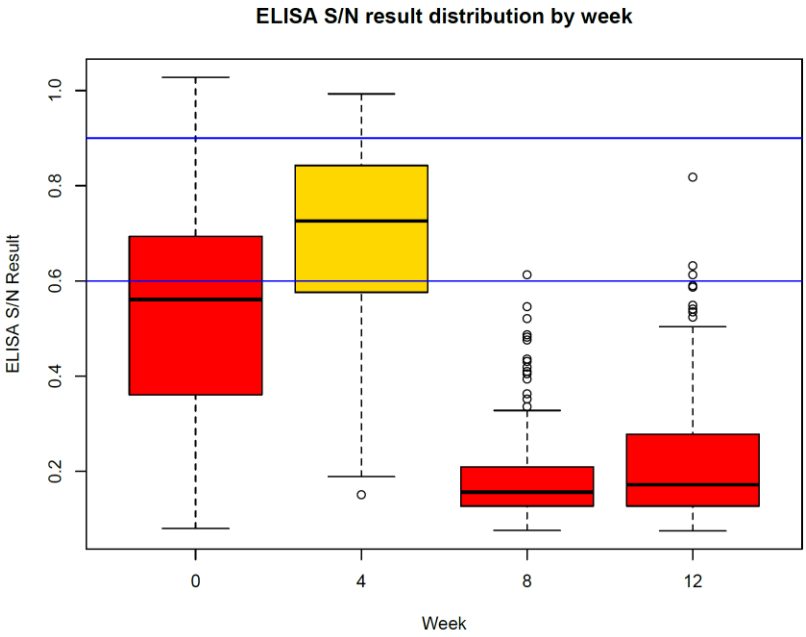


2. **Objective 2: Determine IAV re-infection in endemically infected groups of pigs, with resident viruses:**

a. ELISA results:

Seventy eight percent of samples collected during the 15 wk period were seropositive. Only 6 (4.7%) pigs were seronegative on arrival. The distribution of ELISA results by sampling are shown in Figure 4. IAV seropositives at weeks 0, 4, 8, and 12 ranged between 42% (week 4) and 100% (week 8). There was a significant increase ($p<0.05$) in the S/N mean between week 0 (mean: 0.54, sd:0.23) and week 4 (mean:0.69, sd: 0.19) indicating a loss of maternal antibodies. Although the mean SN result was lower than 0.6 at weeks 8 (mean:0.18, sd:11) and 12 (mean: 0.22, sd: 0.14) with a 100% and 99% of pigs testing positive to IAV respectively, the S/N mean difference between weeks 8 and 12 was statistically different ($p<0.05$).

Figure 3. Distribution of ELISA result to IAV by week sampled. Positive: SN<0.6, suspect: 0.6<SN<0.9, negative: >0.9.



b. IAV re-infection after weaning:

One hundred and fifteen pigs (90.5%) had 2 or more nasal swabs positive to IAV by RT-PCR (CT<40) during the study period. However, only 34 (26.8%) pigs had 2 or more samples positive when a Ct value <35 was used. A pig was considered re-infected if it tested positive in non-consecutive weeks. By this definition, 106 pigs were considered re-infected when a Ct<40 was considered. In contrast, only 30 were considered re-infected when a Ct value <35 was used. Based on sequencing data, most of the pigs that became re-infected, they did so with a different IAV HA subtype (Table 4). However, there were a few pigs that became re-infected with the same HA subtype but with a different H1 cluster. For instance pig 109 became re-infected with H1 cluster 1 in week 1, and cluster 2 in week 14. Pig 128 had cluster 1 and 2 in weeks 3 and 4 respectively. In contrast, pig 116 and 124 became re-infected with the same HA subtype and cluster. Furthermore, pig 1 and 14 became re-infected with

two different subtypes at the same time (Table 4). HA identity comparison within animals that became re-infected with the same subtype and cluster are shown in Table 5.

Table 4. Summary of pigs that became re-infected with IAV by subtype. First column indicates the pig identification number and the first row indicates the week when samples were collected Colored boxes indicate IAV positive swabs by cluster identified in Figure 2. Dark blue: HA cluster 1 (H1). Light blue: HA cluster 2 (H1). Green HA cluster 3 (H3). Red: Mixed infection (cluster 1 (H1) + cluster 3 (H3)).

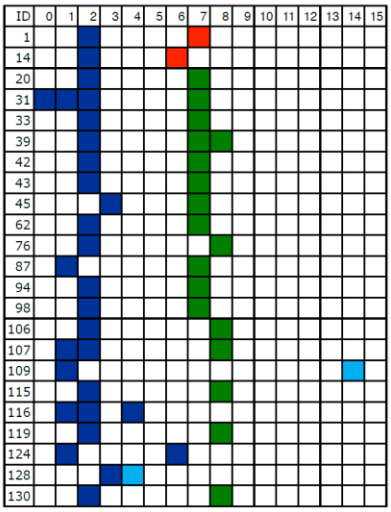


Table 5. Percentage identity between complete HA found in pigs that became re-infected with the same HA subtype and cluster.

Animal	Week comparison	% Identity
1	2 vs 7	99.6
14	2 vs 6	99.5
116	1 vs 2	99.5
116	2 vs 4	98.6
116	1 vs 4	99.1
124	1 vs 6	99.7

Discussion:

Influenza can persist in populations for prolonged periods of time. In this study we evaluated the genetic characteristics of influenza viruses in a wean-to-finish population, assessed the patterns of IAV transmission and evaluated the extent of IAV re-infection to provide some insights into the mechanisms of IAV persistence in populations.

Overall our results indicated that IAV spread rapidly after weaning and that all pigs became infected despite the presence of immunity at weaning. Interestingly there were three genetically distinct

viruses identified in the population and there were two distinct IAV epidemics. The first and second epidemic peaks were dominated by H1 and H3 viruses respectively, although the identification of partial sequences during those peaks indicated the co-circulation of both subtypes during that time. This is interesting since it shows that multiple strains and subtypes can co-circulate within a population at different levels. This observation was possible because sequencing was done using deep sequencing directly from the swabs without prior isolation of the virus in cell culture.

We also showed that a significant percentage of pigs can become re-infected with IAV. In this study, most of the pigs became re-infected with an IAV of a different subtype. However, there were a few pigs that became re-infected with the same subtype but a different strain, or the same strain even though just a few pigs were noted. HA sequences in these pigs shared >99% similarity suggesting that factors, other than the genetics of the virus may play a role at maintaining the virus in populations.

Our results provide a basic understanding of influenza virus diversity and transmission in pigs after weaning in a commercial herd. We identified conditions for IAV persistence and reassortment after weaning. This information is relevant in order to understand why IAV persists in populations and what risk endemically infected populations represent in the generation of new viruses. Our results should assist in the development of better vaccines and strategies to control and reduce the impact of IAV in pigs.

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