

Influenza A virus transmission in swine farms and during transport in the swine supply chain

Jonathan Tin Lai Cheung¹, Eric Lau¹, Ziyang Jin¹, Huachen Zhu¹, Yi Guan¹, and Malik Peire¹

¹The University of Hong Kong School of Public Health

April 25, 2022

Abstract

The last influenza pandemic in 2009 emerged from swine and surveillance of swine influenza is important for pandemic preparedness. Movement of swine during husbandry, trade or marketing for slaughter provide opportunities for transfer and possible genetic reassortment of swine influenza viruses. Over 90% of the swine slaughtered at the central swine abattoir in Hong Kong are imported from farms located in multiple provinces in mainland China. There is opportunity for virus cross-infection during this transport and slaughter process. Of the 26,980 swabs collected in the slaughterhouse in Hong Kong from 5th January 2012 to 15th December 2016, we analyzed sequence data on influenza A (H3N2) virus isolates ($n = 174$) in conjunction with date of sampling and originating farm. Molecular epidemiology provided evidence of virus cross-infection between swine originating from different farms during transport and also evidence of a virus lineage persisting in a swine farm for over 2 years. We used virus serology and isolation data from 4,226 paired pig serum and nasal swabs collected from swine originating from Guangdong Province to compare the force of infection (FOI) during transport and within farms. The mean weekly FOI during transport was $\lambda_t = 0.0286$ (95% CI = 0.0211-0.0391) while the weekly FOI in farms was $\lambda_f = 0.0089$ (95% CI = 0.0084-0.0095), assuming a duration of stay in farm of 28 weeks, suggesting increased force of infection during the transport process. Potential risk factors for infection including the duration in transport, length of stay at slaughterhouse and farm-level seroprevalence were also assessed by multivariable logistic regression analysis. Transport may increase virus cross-infection rates and provide opportunities for virus reassortment potentially increasing zoonotic risk to those involved in the transportation and slaughtering processes.

Influenza A virus transmission in swine farms and during transport in the swine supply chain

Jonathan Tin Lai Cheung¹, Eric HY Lau¹, Ziyang Jin^{1,2,3}, Huachen Zhu^{1,2,3}, Yi Guan^{1,2,3}, Malik Peiris¹

¹ School of Public Health, The University of Hong Kong

² State Key Laboratory of Emerging Infectious Diseases, School of Public Health, The University of Hong Kong, Hong Kong, P. R. China.

³ Guangdong-Hong Kong Joint Laboratory of Emerging Infectious Diseases/Joint Laboratory for International Collaboration in Virology and Emerging Infectious Diseases, Joint Institute of Virology (Shantou University and The University of Hong Kong), Shantou University, Shantou, P. R. China.

Address correspondence to:

Malik Peiris, School of Public Health, The University of Hong Kong, Hong Kong SAR, P.R. China Email: malik@hku.hk

Yi Guan, State Key Laboratory of Emerging Infectious Diseases, School of Public Health, The University of Hong Kong, Hong Kong SAR, P. R. China. Email: yguan@hku.hk

Summary:

The last influenza pandemic in 2009 emerged from swine and surveillance of swine influenza is important for pandemic preparedness. Movement of swine during husbandry, trade or marketing for slaughter provide opportunities for transfer and possible genetic reassortment of swine influenza viruses. Over 90% of the swine slaughtered at the central swine abattoir in Hong Kong are imported from farms located in multiple provinces in mainland China. There is opportunity for virus cross-infection during this transport and slaughter process. Of the 26,980 swabs collected in the slaughterhouse in Hong Kong from 5th January 2012 to 15th December 2016, we analyzed sequence data on influenza A (H3N2) virus isolates ($n = 174$) in conjunction with date of sampling and originating farm. Molecular epidemiology provided evidence of virus cross-infection between swine originating from different farms during transport and also evidence of a virus lineage persisting in a swine farm for over 2 years. We used virus serology and isolation data from 4,226 paired pig serum and nasal swabs collected from swine originating from Guangdong Province to compare the force of infection (FOI) during transport and within farms. The mean weekly FOI during transport was $\lambda_t = 0.0286$ (95% CI = 0.0211-0.0391) while the weekly FOI in farms was $\lambda_f = 0.0089$ (95% CI = 0.0084-0.0095), assuming a duration of stay in farm of 28 weeks, suggesting increased force of infection during the transport process. Potential risk factors for infection including the duration in transport, length of stay at slaughterhouse and farm-level seroprevalence were also assessed by multivariable logistic regression analysis. Transport may increase virus cross-infection rates and provide opportunities for virus reassortment potentially increasing zoonotic risk to those involved in the transportation and slaughtering processes.

(276 words)

Key words: influenza A virus, H3N2, swine, value chains, transmission dynamics, force of infection.

Introduction (Word count: 3910)

Emerging infectious diseases continue to be a major threat to public health. Most emerging or re-emerging infectious diseases are caused by RNA viruses and are zoonotic in origin, arising from domestic livestock or wildlife. Understanding viral transmission dynamics in livestock production and trade pathways can identify potential critical control points that may be amenable to disease control interventions (Leung, 2012).

Swine are an intermediate host in the pathway of pandemic emergence because they express receptors for both avian and human influenza virus in their upper respiratory tract (Ito, 1998). The 2009 H1N1 pandemic virus arose in Mexico through reassortment of gene segments from Eurasian avian-like H1N1 and North American Triple Reassortant swine viruses (Smith, 2009). The two precursor viruses were introduced into Mexico via importation of swine, possibly as breeder-stock. Swine husbandry, trading, marketing and slaughter provide opportunities for the emergence of novel reassortants and zoonotic infections through exposure of humans to swine influenza viruses. An example was H3N2 variant (H3N2v) influenza viruses causing over 300 infections in humans with exposure to swine at agricultural fairs in USA (Jhung, 2013). In Asia, the high densities and close proximity between swine, humans, poultry and wild birds provides an even greater threat for interspecies transmission.

In Hong Kong, over 90% of the swine slaughtered at its abattoirs are imported from mainland China with an annual importation of about 1.45 million swine in 2017 (Centre for Food Safety, 2019). Live pigs for consumption are transported in trucks from the source farms in multiple provinces to a transfer house at the Shenzhen-Hong Kong border where they are held for inspection overnight, and are then transported by trucks or lorries to a centralized abattoir in Hong Kong. Along this transport chain, there is opportunity for virus transmission between consignments of pigs originating from different farms, facilitated by the stress of transport and the densely packed environment. Pigs may also be held for hours to days in holding pens at the abattoir, providing further opportunities for cross infection (details in supporting information, Supplementary figure 1).

A previous study on the transmission dynamics of H1-subtype swine influenza viruses (SIVs) in China (Strelioff, 2013) analyzed virologic and serological data to infer the transmission risk over time. However,

the interaction and cross-reactivity between different lineages of H1 SIVs may have affected interpretation of the serology results from the hemagglutination inhibition (HAI) test. There was a lack of epidemiological information of imported swine population, such as source farm or province and length of stay at abattoir.

Multiple swine influenza viruses (SwIV) are enzootic in swine in China, including classical swine (CS), Eurasian avian-like swine (EA), triple-reassortant swine (TRIG) and pandemic H1N1/2009 (H1N1pdm09) lineage (Vijaykrishna, 2011). Infection with one H1 virus lineage could broaden cross-reactive antibody to other H1-subtype SIVs (Perera, 2011) and hence confound interpretation of sero-epidemiology of H1 swine viruses. There was only one monophyletic H3 lineage (Binh Duong (BD)-like H3N2 lineage) circulating in China since 2010 allowing serology to accurately estimate past infection (Liang, 2014).

The objective of the current study was to understand the transmission dynamics of swine H3N2 viruses at the farm, transport and abattoir stage of the production chain. Focusing on swine H3N2 viruses allowed us to interpret serological data in the context of molecular epidemiology to infer transmission dynamics within the swine production system supplying swine for slaughter in Hong Kong. We compared the risk of infection during transportation and in the farm setting by estimating the force of infection (FOI) and identified the role of factors, such as transport distance and the length of stay at slaughterhouse.

Materials and Methods

Surveillance and sample collection

The Sheung Shui Slaughterhouse is the largest slaughterhouse in Asia, able to accommodate 12,000 pigs and slaughter up to 5,000 pigs per day. From 5th January 2012 to 15th December 2016, our surveillance team collected paired serum and nasal swabs from randomly selected pigs soon after slaughter, 50 paired pig sera and nasal swabs being collected at each visit, which was increased to 75 paired samples since 19th November 2015. Tracheal swabs were also collected at post-mortem inspection. Information of the originating province and farm was derived from pig tattoo identification numbers. We also recorded the arrival date of the slaughtered pigs, from which the length of stay at slaughterhouse was derived. Sampling was conducted twice per month to allow identification of any potential seasonal pattern.

Virus isolation and molecular screening

Swab samples were inoculated into Madin Darby canine kidney (MDCK) cells grown in 24-well plates in minimum essential medium (MEM) supplemented with 2 µg/ml phenylalanyl chloromethyl ketone-treated trypsin (TPCK-trypsin) for virus propagation on the same day and replaced with fresh culture medium on the next day. On day 3 post-inoculation (DPI), viral growth was determined by appearance of cytopathic effects (CPE) in the MDCK cells and by a hemagglutination assay (HA) of cell supernatants using 0.5% turkey red blood cells (TRBCs). The cultures which gave positive results on CPE and HA were further tested using a rapid antigen detection test, DirectigenTM EZ Flu A+B (Becton, Dickinson and Company, New Jersey, USA) to confirm influenza A virus infection. Positive cultures were then passaged on MDCK cells again for a second passage for virus isolation. Positive virus isolates were aliquoted and stored at -80°C.

Viral RNA was extracted from cell culture isolates using QIAmp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). The presence of SIVs was confirmed by a one-step reverse transcription PCR (RT-PCR) targeting the conserved influenza A matrix gene using PrimerScript II High Fidelity RT-PCR Kit (Takara, Japan) (Fouchier, 2000). We adapted a duplex two-step RT-PCR approach for HA and NA subtyping. Complementary DNA (cDNA) was synthesized by reverse transcription using SuperScript III Reverse Transcriptase (InvitrogenTM) with Uni12 primer (Hoffmann, 2001). Gene amplification of HA and NA gene segment was performed using AmpliTaq Gold DNA Polymerase (Thermo Fisher Scientific, Sweden) with subtype-specific primers (Supplementary table 1). PCR products were analysed with 2% agarose gel electrophoresis and virus subtypes were determined.

Serological assays

Serum was extracted from clotted blood samples and stored at -20°C. Hemagglutination inhibition (HAI)

assays were performed on each of the 6,532 serum samples collected, with a representative swine H3 influenza virus strain: A/Swine/Hong Kong/4348/2016 (BD-like H3N2). HAI tests were performed following WHO recommendations for animal influenza diagnosis (World Health Organization, 2011). Serum samples were first treated overnight at 37°C with receptor destroying enzyme (RDE) (Denka Seiken Co Ltd., Tokyo, Japan) and were heat-inactivated for 30 min at 56°C next morning. Serum samples were then serially diluted two-fold (1:10 to 1:1,280) in 96-well plates and 25 µL of virus antigen with four hemagglutination units was added to an equal volume of each serum dilution in duplicate. After 1h incubation, 50µL of 0.5% TRBCs was added to each well and incubated for 30 min. The HAI titer was defined as the highest serum dilution that inhibited complete hemagglutination. For calculation of the geometric mean titer (GMT), serum samples with a titer of <1:10 was assigned a value of 5 and those with [?]1:1,280 were assigned a value of 1:1,280.

Statistical analysis

We fitted a logistic regression model to predict isolation of influenza A(H3N2) subtype, considering factors such as length of stay at slaughterhouse of the live pigs, transport distance from originating farms to Hong Kong, HAI titer against the reference H3N2 strain (A/swine/Hong Kong/4348/2016) of individual pigs, and H3 seroprevalence of the specific originating farms.

The H3 seroprevalence of each originating farm was calculated as the proportion of seropositive (HAI titer [?]1:40) samples in 2012-2016 and only those samples collected from major source farms ([?]20 samples over the study period) were analysed (n = 5,832) (Figure 1). We stratified the individual anti-H3 HAI titer level into sero-negative (<1:40) and sero-positive ([?]1:40).

The slaughtered pigs originated from more than 150 farms. While we had the location of each farm to the level of the Province, exact geo-spatial farm locations were not available. We estimated the transport distance based on the originating province and stratified data into 3 groups: a) nearest, from Shenzhen, Zhuhai and other cities of Guangdong Provinces (within 150km from Hong Kong approximately); b) more distant, from Guangxi, Hainan, Hunan and Jiangxi Provinces (approximately 500-700 km), and c) most distant, from Hubei, Hebei, Henan and Zhejiang Provinces (approximately >900 km). All factors were tested in the multivariable analysis. P value <0.05 was considered statistically significant. All statistical analyses were conducted using R version 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria).

We further estimated the force of infection (FOI) in farms and during transportation of swine respectively, based on the analysis of virological and serological results on H3-subtype SIVs in the subset where paired serum and swab samples were available. Prior to importation of live pigs to Hong Kong, pigs from distant provinces were gathered by local traders but without clear records. Therefore, the exact duration of transportation of individual pigs were not available. Hence, we performed the FOI analysis based on pigs from Guangdong only (n = 4,226, 65% of the total samples) (Figure 1) from which pigs were transported directly to Hong Kong from the farms, to minimise discrepancies between actual and assumed exposure duration.

Paired viral isolation and serological data were utilized to deduce the infection history of individual pigs. Previous studies had reported that pigs would begin shedding virus in nasal swabs by 1-3 days post-exposure and virus shedding would last for 4-5 days (Janke, 2013), whereas circulating antibodies become detectable 10-14 days after infection (Detmer, 2013). The time delay between virus shedding period and seroconversion allowed us to deduce where infections had taken place. Specifically, we defined the number of pigs which have experienced 4 possible infection histories: n_1 , naïve population (negative virological and serological test results); n_2 , recent primary infection (positive virological and negative serological test results); n_3 , previous infection (negative virological and positive serological test results); and n_4 , recent secondary or re-infection (positive virological and serological test results). We estimated the probability of infection during transport (p_t) from the virus isolation rate at the abattoir among swine naïve to H3N2 prior to transportation i.e. sero-negative to H3 antigens, so $p_t = n_2 / (n_1 + n_2)$. The probability of infection in farm (p_f) was estimated by the proportion of seropositive samples, i.e., $p_f = (n_3 + n_4) / (n_1 + n_2 + n_3 + n_4)$, assuming recently infected pigs have not yet seroconverted.

The force of infection (FOI), defined as the instantaneous rate at which a susceptible pig becomes infected,

was used to quantify the transmission potential. The relationship between FOI and probability of infection p is given by:

$$p = 1 - \exp\left(-\int_{t_1}^{t_2} \lambda(t) dt\right)$$

where t_1 , t_2 are the start and end times of the exposure, $\lambda(t)$ is the FOI from infectious pigs in farms or transport system. The probability of infection during transport depends on the duration of travelling time for imported pigs. We assumed the exposure duration related to transport (time before slaughtering, including transportation, inspection and holding) is 3 days. The ages of the pigs sampled were 28 to 36 weeks old (no individual data was recorded) and pigs in farms were assumed to have 4 to 8 weeks of protection by maternal antibodies (Markowska-Daniel, 2011). A previous study on maternally derived antibodies suggested that the maternally acquired immunity may only mask clinical illness of the pigs but not protecting them from infection (Loeffen, 2003). Therefore, we considered possible exposure durations of 20, 28 and 36 weeks for the estimation of FOIs in farm.

Whole-genome sequencing and assembly

The whole-genome sequence of each influenza virus isolate was obtained by using the Genome Sequencer Junior (Roche 454) or ABI 3730 genetic analyzer (Applied Biosystems) as previously described (Vijaykrishna, 2011, Liang, 2014). Original reads that shared >90% sequence identity in a 40-nucleotide overlapping region were assembled into contigs using Lasergene, version 9.0 (DNASTar).

Phylogenetic analysis and molecular epidemiology

Molecular epidemiology was used to define evidence of virus clustering and thus the occurrence of swine-to-swine transmission. Analysis was conducted on full genomes of all H3N2 swine influenza virus isolates sequenced in our study (Figure 1). The influenza virus sequences were analysed together with other existing Asian H3-subtype swine influenza virus sequences retrieved from the NCBI Influenza Virus Resource (Supplementary table 2) (Bao, 2008) and GISAID (Supplementary table 3) (Shu, 2017). All Asian H3 SIVs with a complete set of segments in full-length were included and identical sequences were collapsed. Each genome segment was aligned separately using MAFFT version 7 (Katoh, 2013) and alignments were then trimmed to coding regions. The resultant data sets contained a total of 353 full genome sequence sets.

The coding regions of gene segments PB2, PB1, PA, HA, NP, NA, MP and NS were concatenated using Geneious 2019.2.1 (<http://www.geneious.com>). Multiple alignment for the concatenated whole genomes was performed with MAFFT version 7. We inferred the maximum-likelihood phylogenetic trees using IQ-TREE (Nguyen, 2014) and the built-in ModelFinder (Kalyaanamoorthy, 2017) to determine the best-fit substitution model. Branch supports were obtained using approximate likelihood ratio test (aLRT) (Guindon, 2010) to assess the robustness of the phylogenetic tree. Branch support aLRT statistics were shown at major nodes with values larger or equal to 0.8.

Results

Virus isolation in pigs

During the surveillance period from 2012 to 2016, a total of 8,954 nasal swabs (NS), 18,026 tracheal swabs (TS) and 6,532 pig sera were collected from pigs originated from 505 pig farms in 11 provinces in Mainland China and Hong Kong. The median number of swab samples collected per visit from the Sheung Shui slaughterhouse was 226 (range 102-291). H1N1 ($n = 278$), H3N2 ($n = 174$) and H1N2 ($n = 51$) subtype viruses were isolated. Only H3N2 viruses are further analysed in this study. There was a significantly higher influenza A H3-subtype virus isolation rate in nasal swabs, compared with tracheal swabs ($p = 0.014$).

Phylogenetic analysis and molecular epidemiology

Whole-genome sequencing and phylogenetic analysis were carried out for all 174 H3N2 viruses isolated from both nasal and tracheal swab samples during the study period. Together with 179 reference H3 SIV genome sequences retrieved from the existing databases (GenBank, GISAID), a maximum likelihood phylogenetic tree of 353 concatenated genomes of H3 SIVs was constructed. All tattoo codes indicating farm of origin and sampling dates of the H3 isolates were labelled in the tree (Figure 3). The 174 sequences of the H3N2 virus isolates can be grouped into 9 clusters, numbered 1-9, of closely related virus sublineages within the concatenated phylogenetic tree. Genetically similar viruses were detected in pigs from multiple farms on the same sampling occasion. For example, all H3N2 viruses collected on 3rd July 2014 formed a subcluster of taxa with closely related evolutionary history (subcluster 5). Interestingly, in subcluster 5, 18 nearly identical H3N2 viruses were isolated at the abattoir from pigs originating from 13 different farms in 3 different provinces (Guangdong, Hunan and Jiangxi). Similarly, in subcluster 4, there were 17 highly related H3N2 viruses isolated from pigs originated from 5 different farms in 2 provinces (Jiangxi and Guangdong) on one sampling date 7th January 2016. A similar phenomenon was found in virus subclusters 6, 7 and 8. It was highly improbable that a genetically identical virus was simultaneously circulating in multiple farms and provinces at the same time period. Thus, the phylogeny was strongly suggestive of a virus from a common source which transmitted to swine from other farms during transportation or while being held within the abattoir prior to slaughter.

One of these farms GD101 had similar but not identical viruses isolated on 2nd July 2015 and 7th September 2016, suggesting that this virus sub-lineage was persisting within the farm for over two years.

Seroprevalence of H3N2 influenza virus in pigs

There was only one monophyletic lineage of H3 SIVs isolated in our samples, that being the BD-like H3 lineage. H3N2 seroprevalence was assessed in 6,532 sets of paired nasal swabs and sera. The HAI seroprevalence of BD-like H3 SIVs in this subset was 20.5% (1,340/6,532) with HAI titer [?]:1:40 against the reference H3 strain (A/swine/Hong Kong/4348/2016). The virus isolation rates and seroprevalence of H3N2 in swine were shown in Figure 2. No seasonal pattern was identified.

Multivariable logistic regression on H3-SIV

Of the 6,532 paired nasal swabs and pig sera collected over the study period, 65% of the swine originated from Guangdong province, followed by Jiangxi (17%), with relatively fewer isolates from other provinces (Table 1). We isolated 63 H3N2 viruses from swabs and H3N2 antibodies were detected in 1,340 pigs (Table 1), with 7 pigs having both virus isolates and antibodies. Samples collected from farms ($n = 5,832$) which provided [?]:20 samples were included in our logistic regression. Multivariable logistic regression was fitted to explain H3-subtype virus infections (Table 2).

In the multivariable logistic regression model (Table 2), pigs with anti-H3 HAI titers [?]:1:40 were significantly associated (adjusted OR [aOR] = 0.33, $p = 0.012$) with a lower risk of H3 virus detection in the abattoir (i.e. presumed infection during transportation). This reflected the protection against infection provided by the humoral immune response. Swine from source farms with high H3 seroprevalence ($>30\%$) showed significantly higher H3 virus isolation rates (aOR = 2.24, p -value = 0.015). No significant difference was observed in the virus isolation among pigs with different length of stay at the slaughterhouse and transport distance.

Force of Infection (FOI) in farms and during transport

There were in total 4,226 paired pig sera and nasal swabs collected from swine originating from Guangdong, with 46 (1.09%) H3 viruses isolated and 936 (22.1%) having antibody titers [?]:1:40 (sero-positive) to H3 SIV (Table 1). Among the 3,290 sero-negative samples, there were 40 (1.22%) H3 viruses isolated. The mean weekly FOI λ_t during transport for swine originating from Guangdong was estimated to be 0.0286 (95% CI = 0.0211-0.0391), while the weekly force of infection in farms were $\lambda_f = 0.0125$ (95% CI = 0.0118-0.0134), 0.0089 (95% CI = 0.0084-0.0095), and 0.0069 (95% CI = 0.0065-0.0074) respectively, assuming exposure durations of 20, 28 and 36 weeks in farms. Hence, the relative FOI during transport was 229-414% of that

in farms, indicating a noticeable increased transmission intensity during transportation comparing to that within farms.

Discussion

Swine slaughtered in a large central abattoir in Hong Kong were sourced from farms in multiple provinces of China. We utilized paired virological and serological surveillance data, together with data on farm of origin and date of sampling to assess the transmission risk in the swine transport and marketing system in China. Our results showed that pigs experienced a higher FOI (229%-414%) during transport per-unit time, compared to that within the farms. This suggested that the industrial scale swine production, transportation and marketing of live pigs provide settings favorable to influenza transmission, though the much longer stay in farms allow more infections over swine's lifetime. The phylogenetic tree topology of the concatenated genomes also provided clear molecular epidemiological evidence of cross-transmission along the transport chain, between swine originating from different parts of mainland China. Interestingly, the isolation of similar though not identical viruses from the same farm over a two-year period suggested that swine influenza virus lineages may be maintained within a single source farm for over a year. We did not have detailed farm management data to ascertain herd sizes or management practices, in particular whether these large farms are ever completely emptied of pigs, which was unlikely. The likelihood that sequential batches of swine of different age groups are raised on the same farm increases the possibility of a virus to maintain itself within farms with large herd sizes.

We found that pigs were more likely to be infected with H3 virus during transportation, if originating farms had a high seroprevalence over 30%. The high farm-level seroprevalence implied a high circulation of H3 SIV in farms. Hence, some pigs from the herd were more likely to be infectious at the beginning of the transport chain and more likely to transmit to others along the route (e.g. inspection or holding at the transfer house and holding within the abattoir). However, the infection risk during transportation for pigs with HAI titer of [?]1:40 reduced substantially by 67%, which is compatible with the protective effect of humoral immune response for humans (Hobson, 1972).

Distance of transportation was not found to be associated with the risk of transmission. We observed a lower transmission risk for swine from farms further away from Hong Kong, though the difference was not statistically significant (Table 2). There could be other economic factors which may affect biosecurity measures during transport for these farms. The extent of mixing with other swine during inspection, waiting or holding at the transfer house, quarantine station and abattoir may pose greater transmission risks than that during transportation, with only about 40 pigs in a truck. Hence, the relative contribution of transmission risk during transportation may be less, even with a long trip from source farms to abattoir, than that associated with the mixing taking place at the quarantine stations and other common transport hubs. The isolation rate did not increase significantly for pigs staying longer times in the abattoir in Hong Kong which may imply that transmission within the abattoir was not a major driver of cross-infection. This is possibly related to the short time pigs were held in the abattoir prior to slaughter which may not provide adequate time for transmission and incubation period for there to be detectable virus at slaughter. The emergence of African swine fever in China since 2018 has led to a further shortening of the period of pigs being held in the abattoir in Hong Kong prior to slaughter, likely further reducing risk of cross-infection within the abattoir. The increased FOI during transportation may be facilitated by the high-density and high-stress environment at the transfer houses at the Shenzhen-Hong Kong border. Limiting the number of pigs and time of travel may reduce cross-infection.

There were several limitations in our study. First, the actual duration and distance of transportation duration and distance for the live pigs were not available. We used province as a proxy for duration of transport. We could not rule out impacts arising from other activities related to swine trading. Secondly, while pigs from farms not previously infected with H3N2 virus got infected during the transport to the abattoir, we did not have unequivocal evidence that new viruses were introduced back to farms, although this could occur through contaminated trucks. Third, our estimates focused on H3-subtype SIV only, giving us a smaller sample size. The serological cross-reactivity between multiple co-circulating H1 viruses make such an analysis difficult to

interpret with H1 swine viruses. Finally, our study deduced the infection history of individual pigs based on surveillance in the slaughterhouse only. The actual prevalence of infectious virus in individual farms or transfer house were not available, which prevented us from estimating the transmission risk at different points of transportation.

In conclusion, we used paired serological and virologic samples collected from a swine slaughterhouse to examine the infection history of the swine from farms and during transportation. We found that transport and holding settings provided a favorable environment for swine-to-swine cross transmission per unit time when compared with farms. However, the longer period of time a pig spends on the farm means that the cumulative risk of acquiring infection in the farm is higher. The risk posed by the transport process was co-infection with new viruses which may provide opportunities for virus reassortment providing some risk to those involved in the transportation and slaughtering processes. There may also be the possibility of viruses being introduced back to naïve source farms via fomites and trucks, although this study was not able to investigate this possibility. Further assessment of the relative contribution of influenza transmission at different processes during transportation is needed to identify high risk area for improving disease control.

Acknowledgments: We acknowledge research funding from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under Contract No. 75N93021C00016, HHSN266200700005C and HHSN272201400006C and the Theme Based Research grant (T11-712/19-N) from the Research Grants Council of Hong Kong SAR and the Health Medical Research Fund (Ref no: HKS-15-E02) from the Food and Health Bureau of Hong Kong SAR.

Conflicts of interest: The authors declare no conflicts of interest.

Data availability statement: All data from this study have been disclosed.

Orchid: Malik Peiris ID <https://orcid.org/0000-0001-8217-5995>

Reference:

Bao Y, Bolotov P, Dernovoy D, Kiryutin B, Zaslavsky L, Tatusova T, Ostell J, Lipman D. (2008). The influenza virus resource at the National Center for Biotechnology Information. *J Virol.* 82(2):596-601. doi: 10.1128/JVI.02005-07.

Centre For Food Safety. Slaughterhouses and Disease Surveillance 2019 [Available from: https://www.cfs.gov.hk/english/import/import_sds.html].

Detmer S, Gramer M, Goyal S, Torremorell M, Torrison J. (2013). Diagnostics and Surveillance for Swine Influenza. In: Richt JA, Webby RJ, editors. Swine Influenza. Berlin, Heidelberg: Springer Berlin Heidelberg. p. 85-112.

Fouchier RA, Bestebroer TM, Herfst S, Van Der Kemp L, Rimmelzwaan GF, Osterhaus AD. (2000). Detection of influenza A viruses from different species by PCR amplification of conserved sequences in the matrix gene.

J Clin Microbiol. 38(11):4096-101. doi: 10.1128/JCM.38.11.4096-4101.2000.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol.* 59(3):307-21. doi: 10.1093/sysbio/syq010.

Hobson D, Curry RL, Beare AS, Ward-Gardner A. (1972). The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *J Hyg (Lond).* 70(4):767-77. doi: 10.1017/s0022172400022610. PMID: 4509641; PMCID: PMC2130285.

Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. (2001). Universal primer set for the full-length amplification of all influenza A viruses.

Arch Virol. 146(12):2275-89. doi: 10.1007/s007050170002. PMID: 11811679.

Ito T, Couceiro JN, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG, Kawaoka Y. (1998) Molecular basis for the generation in pigs of influenza A viruses with pandemic potential.

J Virol. 72(9):7367-73. doi: 10.1128/JVI.72.9.7367-7373.1998.

Janke BH. (2013). Clinicopathological Features of Swine Influenza. In: Richt JA, Webby RJ, editors. Swine Influenza. Berlin, Heidelberg: Springer Berlin Heidelberg p. 69-83.

Jhung MA, Epperson S, Biggerstaff M, Allen D, Balish A, Barnes N, Beaudoin A, Berman L, Bido S, Blanton L, Blythe D, Brammer L, D'Mello T, Danila R, Davis W, de Fijter S, Diorio M, Durand LO, Emery S, Fowler B, Garten R, Grant Y, Greenbaum A, Gubareva L, Havers F, Haupt T, House J, Ibrahim S, Jiang V, Jain S, Jernigan D, Kazmierczak J, Klimov A, Lindstrom S, Longenberger A, Lucas P, Lynfield R, McMorro M, Moll M, Morin C, Ostroff S, Page SL, Park SY, Peters S, Quinn C, Reed C, Richards S, Scheftel J, Simwale O, Shu B, Soyemi K, Stauffer J, Steffens C, Su S, Torso L, Uyeki TM, Vetter S, Villanueva J, Wong KK, Shaw M, Bresee JS, Cox N, Finelli L. (2013). Outbreak of variant influenza A(H3N2) virus in the United States. *Clin Infect Dis.* 57(12):1703-12. doi: 10.1093/cid/cit649.

Kalyanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermini LS. (2017). ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat Methods.* 14(6):587-589. doi: 10.1038/nmeth.4285.

Katoh K, Standley DM. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol.* 30(4):772-80. doi: 10.1093/molbev/mst010.

Leung Y, Lau E, Zhang L, Guan Y, Cowling B, Peiris JSM. (2012) Avian Influenza and Ban on Overnight Poultry Storage in Live Poultry Markets, Hong Kong. *Emerging Infectious Diseases.* 18(8):1339-41.

Liang H, Lam TT, Fan X, Chen X, Zeng Y, Zhou J, Duan L, Tse M, Chan CH, Li L, Leung TY, Yip CH, Cheung CL, Zhou B, Smith DK, Poon LL, Peiris M, Guan Y, Zhu H. (2014). Expansion of genotypic diversity and establishment of 2009 H1N1 pandemic-origin internal genes in pigs in China. *J Virol.* 88(18):10864-74. doi: 10.1128/JVI.01327-14.

Loeffen WL, Heinen PP, Bianchi AT, Hunneman WA, Verheijden JH. (2003). Effect of maternally derived antibodies on the clinical signs and immune response in pigs after primary and secondary infection with an influenza H1N1 virus. *Vet Immunol Immunopathol.* 20;92(1-2):23-35. doi: 10.1016/s0165-2427(03)00019-9.

Markowska-Daniel I, Pomorska-Mól M, Pejsak Z. (2011). The influence of age and maternal antibodies on the postvaccinal response against swine influenza viruses in pigs. *Vet Immunol Immunopathol.* 142(1-2): 81-6. doi: 10.1016/j.vetimm.2011.03.019.

Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. (2015). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol.* 32(1):268-74. doi: 10.1093/molbev/msu300.

Perera RA, Riley S, Ma SK, Zhu HC, Guan Y, Peiris JSM. (2011). Seroconversion to pandemic (H1N1) 2009 virus and cross-reactive immunity to other swine influenza viruses. *Emerg Infect Dis.* 17(10):1897-9. doi: 10.3201/eid1710.110629.

Shu Y, McCauley J. (2017). GISAID: Global initiative on sharing all influenza data - from vision to reality. *Euro Surveill.* 30;22(13):30494. doi: 10.2807/1560-7917.ES.2017.22.13.30494.

Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghvani J, Bhatt S, Peiris JS, Guan Y, Rambaut A. (2009). Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature.* 459(7250):1122-5. doi: 10.1038/nature08182. PMID: 19516283.

Streliaoff CC, Vijaykrishna D, Riley S, Guan Y, Peiris JSM, Lloyd-Smith JO. (2013). Inferring patterns of influenza transmission in swine from multiple streams of surveillance data. *Proc Biol Sci.* 280(1762):20130872. doi: 10.1098/rspb.2013.0872.

Vijaykrishna D, Smith GJ, Pybus OG, Zhu H, Bhatt S, Poon LL, Riley S, Bahl J, Ma SK, Cheung CL, Perera RA, Chen H, Shortridge KF, Webby RJ, Webster RG, Guan Y, Peiris JSM. (2011). Long-term evolution and transmission dynamics of swine influenza A virus. *Nature*. 473(7348):519-22. doi: 10.1038/nature10004. PMID: 21614079.

World Health Organization. (2011). Manual for the laboratory diagnosis and virological surveillance of influenza. Geneva, Switzerland: World Health Organization.

Table 1 . Summary of the H3N2 virus isolate and H3 seropositive rates in samples from different origins, sorted by number of samples.

Province of origin	No. of samples	No. of H3 virus isolates	No. (%) H3 seropositive of those tested
Jiangxi	1,072	12	149 (13.9)
Hunan	530	2	108 (20.4)
Hebei	298	0	58 (19.5)
Hubei	170	1	36 (21.2)
Guangxi	110	1	25 (22.7)
Hainan	61	0	17 (27.9)
Henan	28	1	4 (14.3)
Zhejiang	13	0	1 (7.7)
Zhuhai, Guangdong	11	0	3 (27.3)
Shenzhen, Guangdong	13	0	3 (23.1)
Guangdong, other	4,226	46	936 (22.1)
Total	6,532	63 (1%)	1340 (20.5)

Table 2 Summary of the influenza A virus isolation rates in samples with different characteristics

Characteristics	No of samples	No. (%) H3 positive	Adjusted OR	95% confidence intervals	p-value
Length of stay at slaughter-house	Length of stay at slaughter-house	Length of stay at slaughter-house	Length of stay at slaughter-house	Length of stay at slaughter-house	Length of stay at slaughter-house
< 1 day	3446	33 (1.0)	1	Reference	
1-2 days	2183	23 (1.1)	1.30	0.76-2.25	0.339
>2 days	203	2 (1.0)	1.20	0.28-5.08	0.802
Transport distance+	Transport distance+	Transport distance+	Transport distance+	Transport distance+	Transport distance+
Nearest+	3863	45 (1.2)	1	Reference	
More distant+	1519	12 (0.8)	0.77	0.39-1.15	0.449
Most distant+	450	1 (0.2)	0.18	0.02-1.34	0.094
Anti-H3 HAI titer	Anti-H3 HAI titer	Anti-H3 HAI titer	Anti-H3 HAI titer	Anti-H3 HAI titer	Anti-H3 HAI titer
Sero-negative (<1:40)	4625	52 (1.1)	1	Reference	
Sero-positive ([?]1:40)	1207	6 (0.5)	0.33	0.14-0.79	0.012
Sero-prevalence of source farms	Sero-prevalence of source farms	Sero-prevalence of source farms	Sero-prevalence of source farms	Sero-prevalence of source farms	Sero-prevalence of source farms

Characteristics	No of samples	No. (%) H3 positive	Adjusted OR	95% confidence intervals	p-value
Low (<10%)	1963	16 (0.8)	1	Reference	
Moderate (10-30%)	2047	16 (0.8)	1.16	0.57-2.34	0.682
High (>30%)	1822	26 (1.4)	2.25	1.17-4.32	0.150
Total	5832	58 (1.0)			

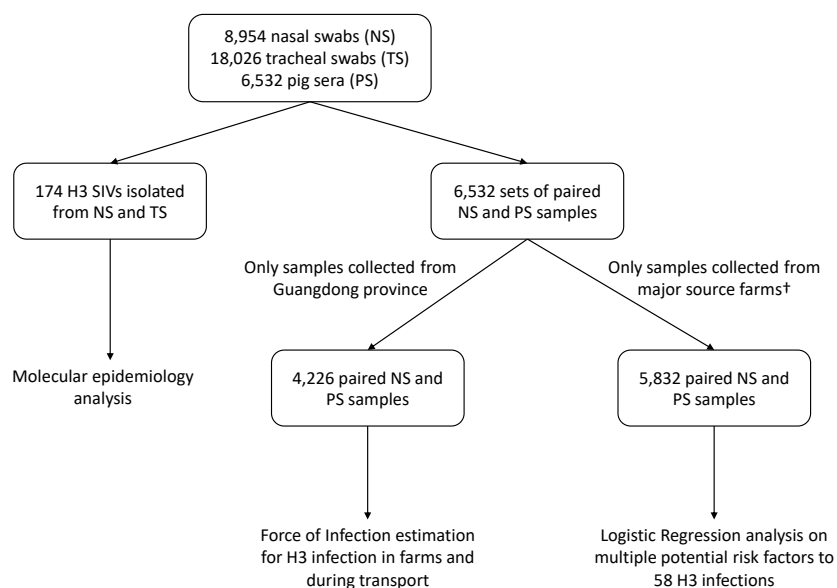
+ Nearest: Guangdong, including Shenzhen and Zhuhai; More distant: Guangxi, Hainan, Hunan, Jiangxi; Most distant: Hubei, Hebei, Henan, Zhejiang.

Figure legends:

Figure 1. Sample collection and plan of analyses.

Figure 2. Monthly H3N2 seropositive and virus isolation rate in 6,532 sets of paired nasal swabs and pig sera, 2012-2016.

Figure 3. Maximum likelihood phylogenetic tree of 174 concatenated genomes of H3N2 swine influenza viruses in 2012-16. The tree was constructed using IQ-TREE and branch supports were obtained with SH-aLRT test. Strain name is followed by pig tattoo codes and sampling date. Scale bar is given in numbers of substitutions per site and branch support aLRT statistics are shown at major nodes with value [?] 0.8.



† Major source farm is defined as farms providing ≥ 20 samples

