Influenza Virus Circulation in Swine of Southern Vietnam, 2009

Vo Minh Hien[1,2], Pham Hong Anh[1], Long Thanh Ngo[3], Than Van Can[4], Thai Quoc Hieu[5], Phan Xuan Thao[6], Nguyen van Dung[6], Hoang Thi Thanh Hang [1], Ngo Thi Hoa[1], Katherine L Anders[1], Jeremy Farrar[1], Do Quang Ha[1], H Rogier van Doorn[1], Juliet E Bryant[1]

[1]Oxford University Clinical Research Unit, Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam; [2]Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam; [3]Regional Animal Health Service, Number 6, Ho Chi Minh City, Vietnam; [4]Sub- Department of Animal Health, Dong Nai, Vietnam; [5] Sub- Department of Animal Health, Tien Giang, Vietnam; [6]Sub- Department of Animal Health, Ho Chi Minh City, Vietnam

ABSTRACT

The zoonotic reservoir of influenza in Vietnamese pigs remains poorly characterized. We initiated influenza surveillance activities among slaughterhouse pigs in southern Vietnam during November and December 2009, shortly after the introduction of pandemic A/H1N1-2009 to Ho Chi Minh City. We wished to determine whether human pandemic A/H1N1-2009 viruses had been transmitted to pigs, and to assess the dominant circulating influenza subtypes within swine populations. Paired nasopharyngeal swabs and sera were collected from 400 pigs (5-7 months old), and were screened by molecular analysis, hemagluttination inhibition (HAI) assay, and two commercial NP Influenza A ELISA tests. None of the swabs tested positive by RTPCR, however the HAI analysis indicated high rates of influenza A exposure, with overall seropositivity of 47%. Subsequently, we screened additional archived pre-pandemic pig sera (dating from 2006-2008) for HAI reactivity to A/H1N1pdm, and demonstrated cross-reactivity between pre-pandemic pig sera and A/H1N1pdm antigen. Our results confirm the widespread circulation of one or more lineages of swine influenza H1 viruses, and underscore the difficulty of interpreting HAI data in the absence of virological confirmation of subtype identity. The porcine reservoirs of influenza in Vietnam require continued monitoring, and there is a need to develop new improved tools for effective surveillance.

Key Words: Swine influenza, Vietnam, Pandemic A/H1N1

---

[1] **For correspondence:**
Juliet E Bryant, PhD
Hospital for Tropical Diseases
Oxford University Clinical Research Unit
190 Ben Ham Tu, District 5
Ho Chi Minh City, Viet Nam
Tel: 84 8 39237954; Fax: 84 8 39238904
jbryant@oucru.org

INTRODUCTION

Swine play an important role in the evolution and ecology of influenza due to their marked susceptibility to cross-species transmission of both human and avian viruses. The tracheal epithelium of pigs contain both sialic acid α2,6 Gal and α2,3 Gal receptors [1], and thus may easily become infected by both human and avian influenza viruses. This susceptibility greatly increases the probability of genetic reassortment, with consequences for host receptor binding affinities, and other determinants of virulence, pathogenicity, and transmissibility. Due to the endemic circulation of A/H5N1 in poultry and wild bird populations in Southeast Asia, and the gravity associated with emergence of avian-pig-human reassortants, ongoing surveillance of influenza within swine is clearly a high priority. This is particularly true of Southeast Asian countries where farming systems are characterized by co-mingling of different species, the density of pig, poultry, and human populations is very high, and human influenza transmission is continuous throughout the year [2].

Unlike the global circulation of human influenza viruses, swine influenza viruses (SIVs) are typically characterized by more restricted distribution patterns, with enzootic foci of different genotypes and distinct lineages in various geographic regions [3]. Three predominant subtypes of influenza virus are prevalent in pigs worldwide: classical swine and avian-like A/H1N1, reassortant H1N2 viruses, and reassortant H3N2 viruses [4]. Current knowledge of the predominant influenza subtypes among Vietnamese pigs is limited to one recent report of a novel SIV reassortant with a human seasonal H3N2 virus [25].

Although human infections with swine influenza can occur, these events remain relatively rare. Worldwide, only 50 cases of human infection with swine influenza were documented between 1970 and 2000, including an outbreak among military recruits in Fort Dix, New Jersey in 1976 [5]. A total of 18 cases have been reported from the USA between 2005 and 2010 [6]. However, several countries throughout the world have now reported human-to-pig transmission of A/H1N1-2009, including Canada [7], Argentina [8], Italy [9] and Thailand [10].

The swine industry of Vietnam is critically important to both food security and the national economy. A better understanding of influenza transmission within swine is important not only to monitor virus evolution and potential zoonotic disease transmission to humans, but also to assess economic and production losses incurred to the industry. Veterinary vaccines are available that might significantly reduce morbidity and improve profitability, and yet influenza vaccination in pigs has never been introduced to Vietnam. Indeed, we surmised that serological investigation of influenza exposure in swine would be feasible due to the absence of vaccination. Here we present results from a survey of influenza in finisher pigs from two provinces of southern Vietnam.

METHODS

Study sites

Samples were collected at randomly selected slaughter facilities in Dong Nai and Tien Giang provinces in Southern Vietnam. Together these two provinces account for a

significant fraction of the swine herd in southern Vietnam, with densities of approximately 1 million head and 500,000 in Dong Nai and Tien Giang, and approximately 47 head per square km [11]. Hog operations in Dong Nai are typically comprised of large commercial facilities with herds of >1000 head in confinement housing. In contrast, farms in Tien Giang are smaller, with open housing and greater mixing between pigs and other domestic animal species [12].

Sampling

A two-stage cluster sampling strategy was used, first to randomly select five slaughter facilities within each of the two provinces, with the probability of selection proportionate to the number of pigs processed. Within each selected slaughter facility, 8 pigs were randomly selected on each of five days, to give a total of 400 market weight healthy pigs (5-7 months olds) from whom swabs and sera were collected. One swab and one blood specimen (5 ml) were collected per pig. Samples were collected immediately after killing and just before rendering. Swabs were placed in Viral Transport Medium (VTM) and stored at room temperature until transfer to 4°C in Department of Animal Health (DAH) laboratories within 24 hrs post sampling, and thereafter held at -80°C. Blood samples were centrifuged and sera aliquoted, then held at -20°C until further processing. All sampling procedures were approved by Regional Animal Health Office Number 6 (RAHO6) and implemented by qualified staff from Department of Animal Health. Information was also collected on the location of the originating farm for each sampled animal.

To further explore the issue of A/H1N1pdm cross-reactivity with other subtypes, we contacted the Sub-Department of Animal Health of Ho Chi Minh City (SubDAH-HCMC) to request access to archived pig sera from routine surveillance activities of the pre-pandemic era (prior to 2009). The SubDAH-HCMC kindly provided 360 sera (120 samples each from 2006, 2007, and 2008). These were screened for HAI reactivity to A/H1N1pdm. With the exception of 15 weaners in 2008, all of the pre-pandemic sera were from healthy mature pigs at least 6 months of age (160 finishers and gilts; 185 sows and boars) sampled on farms located within the metropolitan area of Ho Chi Minh City.

Realtime RT-PCR and viral culture

Swab specimens were pooled (4 swabs/pool) with 200uL per sample, and nucleic acid extractions performed in the automated bioMérieux NucliSENS easyMAG nucleic acid extraction platform (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. Molecular screening for detection of influenza A and swine influenza A viruses was conducted by Real-time RT-PCR following the US CDC protocol [13][14]. Any pools yielding positive RT-PCR were further tested to identify individual positive specimens, and the individual specimens (separate aliquots) were subjected to virus isolation in MDCK cells [14] with a maximum of three passage attempts per specimen.

Serological assays

Sera were tested using two commercial Influenza A NP ELISA kits (Virusys, Maryland USA; and FlockCheck MultiS-Screen antibody test kit, IDEXX Laboratories, Westbrook, ME) [15] according to manufacturer's instructions. In addition, all sera samples were processed by haemagglutination inhibition assay (HAI) using WHO standard protocols [14]. Sera were pretreated using trypsin-heat-

periodate to remove non-specific inhibitors of hemagglutination as per recommendations [14]. The panel of influenza antigens comprised USCDC/ATCC reference reagents for pandemic A/H1N1-2009 (A/California/07/2009 NYMC X-179A) and human seasonal A/H1N1 (A/Brisbane/59/2007 IRV148), and in-house antigen preparations for two contemporary Vietnamese isolates of human seasonal strains (A/Vietnam/1845/2008 (H3N2); B/1401/Vietnam/2008) and one highly pathogenic avian influenza (A/Chicken/Vietnam/LA13/2006 (H5N1)). The in-house antigen preparations were produced in MDCK cell culture[16] and formalin inactivated. Starting sera dilutions for HAI were 1:10, and assays were performed in U-bottom 96-well microtiter plates (Bibby Sterilin Ltd., UK).

Microneutralization assay

To confirm whether HAI positive titers conferred protective immunity against specific virus subtypes, a subset of sera were tested by microneutralization assay (MN) against A/H1N1/Vietnam/9/2009 or A/H5N1/Chicken13/LA-13/2006. MN assays against avian influenza virus were conducted within the biosafety level 3 (BSL3) containment laboratory of Oxford University Clinical Research Unit – Hospital for Tropical Diseases (OUCRU-HTD). Briefly, pretreated sera were serially diluted then mixed with virus at 100 $TCID_{50}$/ 50uL in maintenance medium (MM). 2µg/ml of TPCK-treated Trypsin (Sigma-Aldrich, USA) was added to media for A/H1N1pdm virus but not to the media for A/H5N1. Serum-virus mixtures were incubated for 1 hour at 35°C, 5%CO_2 prior to inoculation in quadruplicate onto confluent monolayers of MDCK cells in 96 well tissue culture plates. Following 3 days of incubation at 35°C and 5%CO_2, plates were scored using microscopic examination of cytopathic effect (+/-). Endpoint neutralization titers were calculated using the Reed and Muench method [14].

Statistical analyses

HAI titers of $\geq$ 1:40 were considered positive [19]. For calculation of geometric mean titer (GMT), values below the detection threshold (1:10) were assigned a value of 5. Statistical analyses were conducted in SPSS version 14 for Windows. Comparison of ELISA and HAI was evaluated by Kappa score. Correlation between microneutralization and HAI was evaluated by Spearman's rho index.

RESULTS

Collections, molecular screening and virus isolation

The 400 pigs sampled at 10 slaughter houses represented 80 different farms located in 59 wards and 16 districts of Tien Giang and Dong Nai. Samples from three pigs were omitted from analysis due to mislabeling. Five of the 99 pooled swab samples were marginally positive by RT-PCR for influenza A. The 20 individual swabs from these 5 pools were retested individually by RT-PCR and subjected to virus isolation. All of the individual swabs tested negative by RT-PCR, with the exception of one swab yielding a Ct value of 39.9 for Swine influenza A. None of the 20 swabs were culture positive (3 serial passage attempts in MDCK cells).

Hemagluttination inhibition (HAI) and microneutralization (MN) assays

Pig sera from both Tien Giang and Dong Nai showed high levels of seropositivity to influenza A (47%, 187/ 397). Reactivity against A/H1N1pdm appeared to be significantly higher than for other antigens tested (Table 1), with 45.6% of sera

(181/397) testing positive for A/H1N1pdm, followed by A/H3N2 at 9.8% (39/397), and A/H5N1 at 0.3%, (1/397). All pig sera were seronegative to human seasonal H1N1 antigen. 69 of the 397 sera (17%) tested positive to both A/H1N1pdm and A/H3N2. In addition to influenza A seroreactivity, four sera exhibited low level (<1:40) reactivity against influenza B. Seroprevalence rates in Dong Nai (56%) were significantly higher than in Tien Giang (35%) (p<.001). The two provinces showed similar predominance of A/H1N1pdm seroreactivity as compared to the other subtypes.

Table 1. Hemagluttination Inhibition titers of healthy pig sera tested in parallel against five influenza antigens of different subtypes.

		A/H1N1pdm			human seasonal H1N1			H3N2			H5N1			Influenza B		
	No. tested	positive	%	GMT	positive	%	GMT	positive	%	GMT	positive	%	GMT	positive	%	GMT
Dong Nai	200	112	56.0%	35.7	0	0.0%	5.0	27	13.5%	12.7	0	0.0%	5.0	4	2.0%	5.5
Tien Giang	197	69	35.0%	25.3	0	0.0%	5.1	12	6.1%	13.0	1	0.5%	5.1	0	0.0%	5.3
Total	397	181	45.6%	30.1	0	0.0%	5.1	39	9.8%	12.9	1	0.3%	5.1	4	1.0%	5.4

Microneutralization assays against A/H1N1pdm and A/H5N1 were performed on 8 of the HAI-seropositive sera. Each of the samples exhibited positive neutralizing titers ≥ 1:158 to A/H1N1pdm, whereas none were able to neutralize A/H5N1. HAI and MN titers for A/H1N1pdm were strongly correlated (Spearman's rho= 0.923-1, p<0.001).

ELISA detection of influenza A antibodies

The two commercial influenza A ELISA tests showed good concordance to one another (Kappa index of 0.780 (p<0.001, CI=0.720-0.840)) (**Table 2**). NP ELISA detected an additional 44 positives over the IDEXX kits, resulting in overall detection rates of 58% (231/397) and 47% (187/397) for NP and IDEXX kits, respectively. The NP-ELISA and IDEXX ELISA results each showed moderate agreement with HAI (Table 2).

Table 2. Correlation between HAI and ELISA assays. Individual serum samples were considered positive for influenza A if had titer ≥ 1:40 to any of tested subtypes. A) NP versus IDEXX ELISA; B) HAI influenza A versus IDEXX; C) HAI influenza A versus NP ELISA.

A		IDEXX ELISA		
		Positive	**Negative**	**Total**
NP ELISA	**Positive**	187 (47%)	44 (11%)	**231 (58%)**
	Negative	0 (0%)	166 (42%)	**166 (42%)**
		187 (47%)	210 (53%)	**397 (100%)**

Kappa index= 0.780 (p<0.001, CI=0.720-0.840)

B		IDEXX ELISA		
		Positive	**Negative**	**Total**
HAI with flu A	**Positive**	148 (37%)	39 (10%)	**187 (47%)**
	Negative	39 (37%)	171 (43%)	**210 (53%)**
	Total	187 (47%)	210 (53%)	**397 (100%)**

Kappa index= 0.606 (p<0.001, CI=0.526-0.686)

C		NP ELISA		
		Positive	**Negative**	**Total**
HAI with flu A	**Positive**	164 (41%)	23 (6%)	**187 (47%)**
	Negative	67 (17%)	143 (36%)	**210 (53%)**
	Total	231 (58%)	166 (42%)	**397 (100%)**

Kappa index= 0.551 (p<0.001, CI=0.469-0.633)

HAI screening of additional pig sera from pre-pandemic era

A total of 360 pre-pandemic sera (dating from 2006, 2007, and 2008) were screened by HAI for reactivity to A/H1N1pdm antigen. Significant reactivity against A/H1N1pdm antigen was observed: 38% positive for 2006 sera; 63% positive for 2007 sera; and 42% positive for 2008 sera.

DISCUSSION

The phenomenon of bidirectional transmission of influenza viruses between pigs and humans is at least partially responsible for the extraordinary complexity of influenza ecology and evolution. Documented human infections with swine-origin influenza viruses remain relatively rare [5], however introductions of human viruses into pig

populations appear to occur rather more frequently [20, 21], and have caused serious outbreaks of swine disease [22]. Persistence of human-origin viruses within swine reservoirs is a significant public health concern, as it greatly augments the epizootic potential of reassortment events. Monitoring the diverse array of influenza viruses within swine is exceedingly difficult, even for resource-rich countries with well-developed programs of systematic animal disease surveillance.

Unfortunately, our survey was unable to recover any virus isolates or sequence data to clarify which influenza subtypes are circulating among pigs of southern Vietnam. However, the serologic data confirmed high rates of influenza A exposure, with significant variation between the two provinces studied. The difference in seroprevalence rates between Dong Nai and Tien Giang suggests significant geographic variation in influenza exposure, perhaps relating to farm size and/or farming practices (e.g. biosecurity). Higher seroprevalence was observed in Dong Nai, where pigs are typically maintained in larger commercial facilities with herds of >1 000 head in confinement housing. The fact that sera dating from prior to 2009 tested positive by HAI against A/H1N1pdm complicates the interpretation of results. Other researchers have previously demonstrated cross-reactivity between avian-like A/H1N1, classical swine A/H1N1 viruses, and A/H1N1pdm by HAI assay [28], as well as neutralizing cross-protection [26]. Clearly there may be multiple different lineages of avian-like, swine-like and human-like H1 viruses circulating within the region, and further clarification will require additional investments in virological surveillance.

Regarding highly pathogenic avian influenza (HPAI), and the possible role of swine in transmission of A/H5N1, both China [29] and Indonesia [30] have now documented evidence of natural infections of A/H5N1 in pigs. Susceptibility of pigs to A/H5N1 appears to be limited, however, and infections may be asymptomatic [31]. Experimental studies of A/H5N1 in pigs indicate clear barriers to transmission [32]. The only previously published work on A/H5N1 influenza among Vietnamese swine was a 2004 sero-epidemiological study in the north that focused exclusively on A/H5N1, and showed positive neutralizing titer to A/H5N1 in 8 of 3175 (0.25%) pigs [33]. Our results confirm that, despite the endemicity of A/H5N1 in chickens and ducks of the Mekong, pig infections with A/H5N1 appear to be extremely rare.

We used two commercial ELISA kits designed to detect influenza A exposure, that have previously been validated to detect antibodies against swine and avian influenza viruses, including exposure to A/H1N1pdm [34-36]. We document a relative sensitivity of Virusys NP-ELISA and IDEXX ELISA kits in comparison to HAI assay of 87.7% and 79.1%, respectively. Discrepancies in test results are likely due to differences in the NP-specific immunogenic response detected by ELISA, as opposed to the full complement of agglutinating antibodies detected by HAI.

The frequency and consequences of virus exchange between human and pig populations remains unclear, however it would seem that heightened surveillance activities in pigs are warranted. Our study underscores the challenge of monitoring currently circulating influenza subtypes in pigs based on serological evidence alone, and the urgent need for development of improved screening technologies. We are currently evaluating a new approach to serological analysis using recombinant HA antigens with much reduced cross-reactivity profiles that is implemented in a high

throughput microarray technology platform. Such approaches will be required to keep pace with rapidly evolving influenza A viruses, and the constantly changing array of viruses within animal reservoirs.

ACKNOWLEDGEMENTS

This research was supported by the Wellcome Trust Major Overseas Programme. Many thanks to the staff of the Subdepartments of Animal Health of Tien Giang, Dong Nai, and Ho Chi Minh City for their assistance in sample collection.

REFERENCES

1. Ito, T., et al., *Molecular basis for the generation in pigs of influenza A viruses with pandemic potential.* J Virol, 1998. **72**(9): p. 7367-73.
2. Nguyen, H.T., et al., *National influenza surveillance in Vietnam, 2006-2007.* Vaccine, 2009. **28**(2): p. 398-402.
3. Smith, G.J., et al., *Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic.* Nature, 2009. **459**(7250): p. 1122-5.
4. Thacker, E. and B. Janke, *Swine influenza virus: zoonotic potential and vaccination strategies for the control of avian and swine influenzas.* J Infect Dis, 2008. **197 Suppl 1**: p. S19-24.
5. Myers, K.P., C.W. Olsen, and G.C. Gray, *Cases of swine influenza in humans: a review of the literature.* Clin Infect Dis, 2007. **44**(8): p. 1084-8.
6. ProMED-mail, *SWINE ORIGIN (TRIPLE REASSORTANT) H3N2 VIRUSES.* 12 Nov 2010.
7. Pasma, T. and T. Joseph, *Pandemic (H1N1) 2009 infection in swine herds, Manitoba, Canada.* Emerg Infect Dis, 2010. **16**(4): p. 706-8.
8. Pereda, A., et al., *Pandemic (H1N1) 2009 outbreak on pig farm, Argentina.* Emerg Infect Dis, 2010. **16**(2): p. 304-7.
9. Moreno, A., et al., *First Pandemic H1N1 Outbreak from a Pig Farm in Italy.* Open Virol J, 2010. **4**: p. 52-6.
10. Sreta, D., et al., *Pandemic (H1N1) 2009 virus on commercial swine farm, Thailand.* Emerg Infect Dis, 2010. **16**(10): p. 1587-90.
11. Organization, F.a.A., *Agricultural Atlas of Viet Nam. A Depiction of the 2001 Rural Agriculture and Fisheries Census. Pro-Poor Livestock Policy Initiative (PPLPI), (www.fao.org/ag/pplpi.html).* 2002.
12. Lemke, U., et al., *Pig production in Vietnam – A review.* CAB Reviews: Perspectives in agriculture, veterinary science, nutrition and natural resources. 3, No. 023 2008
13. WHO, *CDC protocol of realtime RTPCR for influenza A (H1N1).* 2009 April.
14. WHO, *CDS/CSR/NCS/2002.5 – WHO Animal influenza manual, (http://www.who.int/vaccine_research/diseases/influenza/WHO_manual_on_animal-diagnosis_and_surveillance_2002_5.pdf).* 2002.
15. Leuwerke, B., et al., *Comparison of three serological assays to determine the cross-reactivity of antibodies from eight genetically diverse U.S. swine influenza viruses.* J Vet Diagn Invest, 2008. **20**(4): p. 426-32.
16. WHO, *CDC Realtime RTPCR (rRTPCR) Protocol for Detection and Characterization of Influenza.* April 4, 2006.

17. AP, K., P. MS, and S. J., *Concepts and procedures for laboratory-based influenza surveillance.* Atlanta, GA: US Department of Health and Human Services, CDC, 1982.
18. Simmons, C.P., et al., *Prophylactic and therapeutic efficacy of human monoclonal antibodies against H5N1 influenza.* PLoS Med, 2007. **4**(5): p. e178.
19. Song, M.S., et al., *Evidence of human-to-swine transmission of the pandemic (H1N1) 2009 influenza virus in South Korea.* J Clin Microbiol, 2010 Sep. **48**(9): p. 3204-11.
20. Yu, H., et al., *Further evidence for infection of pigs with human-like H1N1 influenza viruses in China.* Virus Res, 2009. **140**(1-2): p. 85-90.
21. Cong, Y., et al., *Reassortant between human-Like H3N2 and avian H5 subtype influenza A viruses in pigs: a potential public health risk.* PLoS One, 2010. **5**(9): p. e12591.
22. Webby, R.J., et al., *Evolution of swine H3N2 influenza viruses in the United States.* J Virol, 2000. **74**(18): p. 8243-51.
23. Brown, I.H., P.A. Harris, and D.J. Alexander, *Serological studies of influenza viruses in pigs in Great Britain 1991-2.* Epidemiol Infect, 1995. **114**(3): p. 511-20.
24. Dalva A. P. Mancini, E.M.S.C., Rita M. Z. Mendonça, Andrea L. F. Dias, Alessandra F. de Castro, José R. Pinto, Ronaldo Z. Mendonça, *Evidence of swine respiratory infection by influenze viruses in Brazil.* Virus Reviews & Research 11 (1-2):39-43., 2006.
25. Ngo, L.T., et al., *Isolation of novel triple-reassortant swine H3N2 influenza viruses possessing the hemagglutinin and neuraminidase genes of a seasonal influenza virus in Vietnam in 2010.* Influenza Other Respi Viruses, 2011.
26. Durrwald, R., et al., *Swine influenza A vaccines, pandemic (H1N1) 2009 virus, and cross-reactivity.* Emerg Infect Dis, 2010 Jun. **16**(6): p. 1029-30.
27. Sreta, D., et al., *Pathogenesis of swine influenza virus (Thai isolates) in weanling pigs: an experimental trial.* Virol J, 2009. **6**: p. 34.
28. Kyriakis, C.S., et al., *Serologic cross-reactivity with pandemic (H1N1) 2009 virus in pigs, Europe.* Emerg Infect Dis, 2010 Jan. **16**(1): p. 96-9.
29. Shi, W.F., et al., *Genetic analysis of four porcine avian influenza viruses isolated from Shandong, China.* Arch Virol, 2008. **153**(1): p. 211-7.
30. Takano, R., et al., *A comparison of the pathogenicity of avian and swine H5N1 influenza viruses in Indonesia.* Arch Virol, 2009. **154**(4): p. 677-81.
31. Nidom, C.A., et al., *Influenza A (H5N1) viruses from pigs, Indonesia.* Emerg Infect Dis, 2010. **16**(10): p. 1515-23.
32. Lipatov, A.S., et al., *Domestic pigs have low susceptibility to H5N1 highly pathogenic avian influenza viruses.* PLoS Pathog, 2008. **4**(7): p. e1000102.
33. Choi, Y.K., et al., *Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004.* J Virol, 2005. **79**(16): p. 10821-5.
34. Ciacci-Zanella, J.R., et al., *Detection of anti-influenza A nucleoprotein antibodies in pigs using a commercial influenza epitope-blocking enzyme-linked immunosorbent assay developed for avian species.* J Vet Diagn Invest, 2010. **22**(1): p. 3-9.
35. de Boer, G.F., W. Back, and A.D. Osterhaus, *An ELISA for detection of antibodies against influenza A nucleoprotein in humans and various animal species.* Arch Virol, 1990. **115**(1-2): p. 47-61.

36. Brown, J.D., et al., *Evaluation of a commercial blocking enzyme-linked immunosorbent assay to detect avian influenza virus antibodies in multiple experimentally infected avian species.* Clin Vaccine Immunol, 2009. **16**(6): p. 824-9.