Swine influenza: clinical, serological, pathological, and virological cross-sectional studies in nine farms in Argentina

Marina Dibárbora,[a] Javier Cappuccio,[b] Valeria Olivera,[a] Maria Quiroga,[b] Mariana Machuca,[b] Carlos Perfumo,[b] Daniel Pérez,[c] Ariel Pereda[a]

[a]Laboratorio Aves y Porcinos, Instituto de Virología CICVyA – Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires, Argentina. [b]Cátedra de Patología Especial, Facultad de Cs. Veterinarias, Universidad Nacional de La Plata, Buenos Aires, Argentina. [c]Virginia-Maryland Regional College of Veterinary Medicine and Department of Veterinary Medicine, University of Maryland, College Park, MD, USA.
Correspondence: Marina Dibárbora, Laboratorio Aves y Porcinos, Instituto de Virología CICVyA – Instituto Nacional de Tecnología Agropecuaria (INTA), CC25 (1712) Castelar, Buenos Aires, Argentina. E-mail: mdibarbora@cnia.inta.gov.ar

ABSTRACT

Background Influenza A viruses (IAV) are important pathogens responsible for economic losses in the swine industry and represent a threat to public health. In Argentina, clinical, pathological, and virological findings suggest that IAV infection is widespread among pig farms. In addition, several subtypes of IAV, such as pH1N1, H3N2, δ1H1N1, and δ2H1N2, have been reported.

Objectives To evaluate the infection patterns of influenza virus in nine pig farms in Argentina.

Methods Clinical, serological, pathological, and virological cross-sectional studies were conducted.

Results Clinical and pathological results were characteristic of endemic influenza infection in eight of the nine farms studied. By rRT-PCR, six of the nine farms were positive to influenza. Five IAV were obtained. Genome analysis determined that four of the isolations were pH1N1 and that the remaining one was a reassortant human origin H3N2 virus containing pandemic internal genes. Serological results showed that all farms were positive to influenza A antibodies. Moreover, the hemagglutination inhibition test showed that infection with viruses containing HA's from different subtypes (pH1, δ1H1, δ2H1, and H3) is present among the farms studied and that coinfections with two or more subtypes were present in 80.5% of positive pigs.

Conclusions Because vaccines against IAV are not licensed in Argentina, these results reflect the situation of IAV infection in non-vaccinated herds. This study provides more information about the circulation and characteristics of IAV in a poorly surveyed region. This study provides more data that will be used to evaluate the tools necessary to control this disease.

Keywords Argentina, influenza, pathology, serology, swine, virology.

Introduction

Influenza A viruses (IAV) are important pathogens responsible for economic losses in the swine industry and represent a constant threat to public health.[1] The clinical presentations of IAV infection in naïve swine populations are associated with outbreaks of acute respiratory disease in which morbidity can reach 100%. Thereafter, an enzootic or subclinical form of infection can be established.[1–4] Virological, serological, and pathological cross-sectional studies are essential to determine the epidemiological status of a farm, region or country.[1]

During the 2009, clinical disease and virus isolation of a pandemic H1N1 virus (pH1N1), in a commercial swine farm were reported for the first time in Argentina.[5] Furthermore, a non-contemporary wholly human H3N2 subtype was isolated from a swine farm and experimental infection showed high transmissibility among pigs.[6] Later, in 2011, reassortants of pH1N1 with H1N2 and H1N1 of human origin have been found.[7] Clinical, pathological, and virological findings suggest that influenza virus infection is widespread among pig farms in Argentina.[8]

The aim of this study was to evaluate the infection patterns of influenza virus in nine pig farms of Argentina with

previous reports of influenza–like signs by clinical, serological, virological, and pathological cross-sectional studies.

Materials and methods

Study design

A cross-sectional study was conducted between January and May 2012. Farm and pig selection criteria in each farm were based on accessibility and convenience as described below:

1. *Herd selection*: Farms with previous reports of influenza-like infection were invited to participate in the study. Nine farms with a total of 21 180 sows, which represents about 10% of the breeder stock of Argentina, accepted to participate in the study. The farms were located in Buenos Aires (two farms), Santa Fe (two farms), Cordoba (four farms), and San Luis (one farm) provinces, which represent the four main swine production areas in Argentina (Table 1).

2. *Pig selection*: Pigs were evaluated to detect influenza-like clinical signs and to measure rectal temperature. Pigs with clinical signs were sampled; however, if <30 pigs with clinical signs were detected in each age group, a random sampling scheme was applied.

Sampling scheme

Nasal swabs and blood samples were obtained from 15 sows, 15 gilts and 30 pigs of 7, 21, 35, 49, 63, 77, 100, and 160 days old (n = 270), from each farm. This sample number, which was calculated using the EpiInfo[tm] software package (CDC, Atlanta, GA, USA), allows us to estimate the prevalence in a population of 1000 or more animals with an estimated prevalence between 5–20% and 95% of confidence.

Serological studies

The ID Screen Influenza A antibody competition ELISA kit (IDVet, Montpellier, France) was performed on sera from pigs according to the manufacturers' instructions. IAV-

positive serum samples from sows and 160-day-old pigs were analyzed for the hemagglutination inhibition (HI) test. The homologous and cross-HI assays were performed separately, using IAV subtypes previously isolated in Argentina: H1N1 cluster pandemic (pH1), rH1N2 cluster delta 1 (δ1H1), rH1N1 cluster delta 2 (δ2H1), and H3N2 cluster 2 (H3). The tests were performed according to standard procedures of Office International des Epizooties.[9] The Geometric Mean Titer (GMT) was calculated for each farm.

Virological and molecular studies

Nasal samples were individually collected with dacron swabs and stored in viral transport medium. Samples were tested in pools of up to five or six swabs collected from pigs from a single age group. Viral RNA was extracted from pooled nasal swabs and lung macerate supernatant using a QIAampViralRNA Mini kit (Qiagen, Hilden, Germany) and used for real-time RT-PCR (rRT-PCR) to detect the M gene of IAV.[10] PCR was performed in an ABIPrism 7500 SDS apparatus (Applied Biosystems™, Foster City, CA, USA). Positive pools by rRT-PCR were opened, and each individual sample was inoculated in Mardin-Darby Canine Kidney cells (MDCK) as described previously.[7]

Genetic analysis and phylogenetic characterization

Viral RNA was extracted from the culture supernatant and used to amplify the complete viral genome of IAV.[11] Sequencing was performed using a BigDye Terminator Kit (Applied Biosystems™) on an ABI 3500 (Applied Biosystems™) using an appropriate set of primers. Sequences were edited and analyzed with BioEdit© (Ibis Biosciences, Carlsbad, CA, USA). The complete genome of each isolate was used for Nucleotide Blast analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the most closely related IAV for each segment. Phylogenetic analyses were conducted using mega (ver. 5.0) software.

Histopathological and immunohistochemical studies

Complete necropsies were performed on pigs found dead during the visit (four farms). Several tissue samples including lung samples were collected for histopathological and virological studies. Samples were fixed in 10% buffered formalin, embedded in paraffin and stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed on tissue of suspected cases using anti-NP antibody as described previously[5]

Results

Influenza-like signs characteristic of endemic influenza infection, such as cough, dyspnea, and fever, were observed in eight of the nine farms studied (Table 1).

Table 1. Farm location, number of sows, production characteristics, and influenza-like clinical signs observed

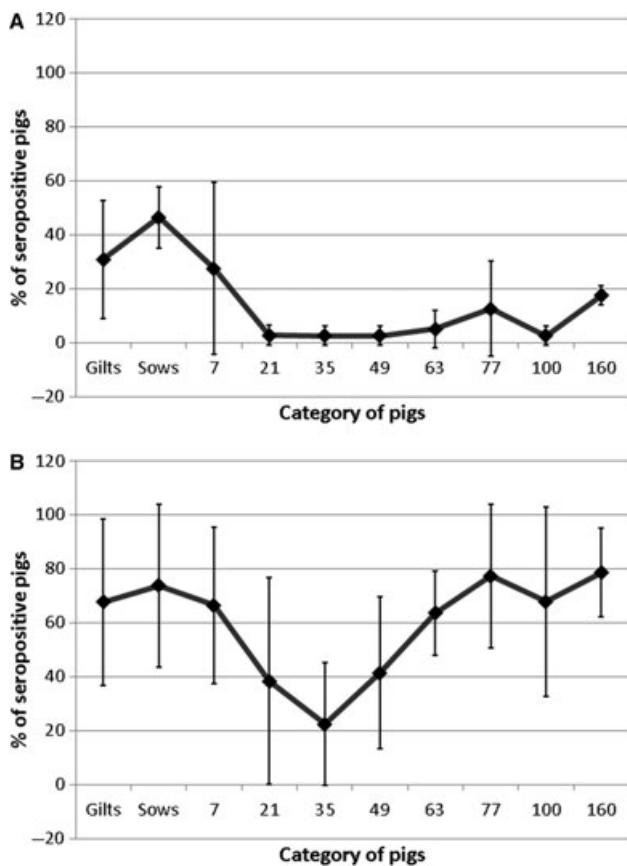| ID	Province	No. sows	Multiple site/one site	Influenza-like signs (days old)
G0	Buenos Aires	6000	3 site	63
G1	Córdoba	740	3 site	Not detected
G2	Santa Fé	2400	3 site	49–63
G3	Córdoba	500	1 site	35–49
G4	Santa Fé	4500	3 site	21 and 140
G5	Córdoba	500	1 site	50 and 120
G6	Buenos Aires	340	1 site	25
G7	San Luis	4500	3 site	35
G8	Córdoba	1700	3 site	35

Figure 1. Two different patterns of influenza A virus infection obtained by ELISA in nine commercial swine farms. Panel A: represents farms with active circulation only in the breeding stock. Panel B: farms with persistent circulation. Graphics show the mean percentage of positive pigs at each age sampled (bars represent the standard deviation).

Figure 2. HI: GMT in sows and fatteners of each farm. Test were performed against PdmH1, δ1H1, δ2H1 and H3 subtypes previously isolated in Argentina.

Serology

Every farm tested positive for IAV antibodies. Overall within-farm seroprevalence by ELISA was of 48·5% and ranged from 7·1 to 79·4. Sows and 160-day-old fatteners showed the highest mean percentage of positivity. However, the range of positive animals varied among farms. The pattern of infection was grouped within two different scenarios. In farms G1 and G3 (Figure 1A), <50% of the sows or gilts were seropositive, and in the rest of the studied categories, the highest percentage of positive pigs was of 20%. In the remaining seven farms (Figure 1B), the mean seropositivity of the breeding stock was 60% or higher. A decrease in antibody levels was observed between 21- and 35-day-old pigs, in concordance with the post-weaning period and then increased steadily during the growing and fattening periods. No correlation was observed between percentage of seropositive pigs, clinical signs, and virological detection from nasal swabs.

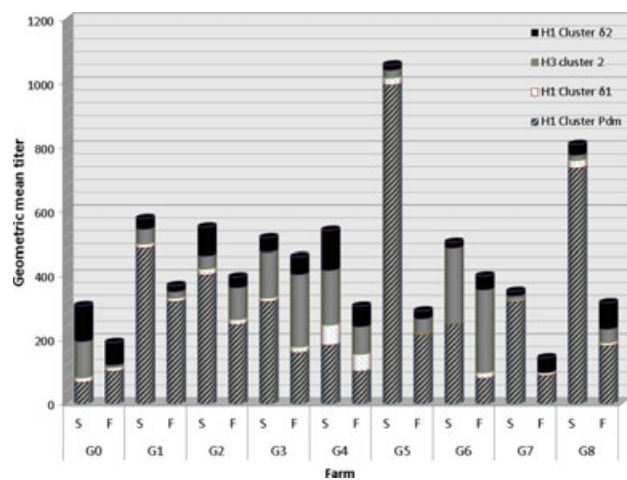In all the farms analyzed, antibodies with reactivity against pH1, δ2H1, and H3 antigens were detected, and in eight of the nine farms antibodies with reactivity against δ1H1 were detected. The GMT was higher in sows than in 160-day-old pigs. The GMT was higher against pH1 antigen than against other antigenic clusters or strains. Only one farm (G0) had higher GMT against H3 than to pH1 antigens in sows, and two farms (G3 and G6) showed the same profile in fatteners. (Figure 2). Moreover, 80·5% of the sera evaluated had antibodies against more than one subtype, in which the most common combination of antibodies were against pH1, δ2H1, and H3, and pH1 and H3 antigens (Table 2).

Virology

Influenza virus was detected from nasal swabs in six of the nine farms (G0, G2, G3, G4, G7, and G8). A total of 33 (8·14%) of the 405 pooled samples analyzed were positive by rRT-PCR. In addition, four of the twelve lung samples with pneumonic lesions, belonging to four different farms, were positive by rRT-PCR. Seventeen virus isolates (51·51%) were obtained from five farms. Genomic characterization of HA, M, and NA genes of all the viruses isolated was carried out. The results showed more than 99% of similarity of these three genes between the isolations, and then we selected only one isolate from each farm as a representative to be fully sequenced. Four of the isolates showed 99% similarity with nucleotide sequences of H1N1 strains. Only one isolate was characterized as a reassortant H3N2 with internal genes of pH1N1 and external genes of human H3N2 (GenBank accession numbers from KC876520 to KC876559). Phylogenetic characterization showed that all the H1N1 isolates clustered together with pandemic viruses and the H3N2 isolate grouped into cluster 2 of the H3N2 subtype (data shown as additional supporting information).

Pathological studies

Thirty-four necropsies and histopathological studies were performed. Twelve pigs had macroscopic pneumonic lung

Table 2. Hemagglutination Inhibition test. Number of sera and percentage of reactivity against different HA subtype antigens of all the ELISA positive sera from sows and 160-day-old pigs (Fatteners)

Subtype	No. Sows	% Sows	No. Fatteners	% Fatteners	Total	Total%
H1pdm + H1δ2 + H3	21	31·8	39	31·4	60	31·6
H1pdm + H3	17	25·7	21	16·9	38	20·0
H1pdm + H1δ1 + H1δ2 + H3	12	18·2	15	12·1	27	14·2
H1pdm	9	13·6	14	11·3	23	12·1
H1pdm + H1δ2	2	3·0	26	20·1	28	14·7
Others	5	7·7	9	8·2	14	7·4
Total ELISA positive sera	66	100	124	100	190	100

lesions. The most common lesion, cranioventral bronchopneumonia, was observed in 10 cases (83·33%), whereas distinctive scattered, dark red foci of lobular consolidation (chessboard-like) were observed in other two cases. Characteristic microscopic lesions such as necrotizing bronchiolitis and small and medium airways denuded or lined with regenerated epithelia and plugged with inflammatory and necrotic epithelial cells were observed in eight of the twelve pigs (66·66%). Immunohistochemistry showed a positive reaction for IAV nucleoprotein only in one case, despite the positive virological results.

Discussion

Influenza A infection seems to be widespread among Argentinean pig farms although caution is exercised when extrapolating the results of this study to the complete Argentinean pig population due to the limited number of farms analyzed and the inclusion criteria. These results are in agreement with previous serological studies in which infection was detected in a high percentage of the farms evaluated.[12] However, no clinical signs or virus isolation were observed or reported before 2009.[5] Clinical signs observed in this study are similar to those reported in other studies, which mentioned that subclinical or endemic presentations are common.[3,4] However, in several farms, managers reported a loss on productivity output associated with an increase in the percentage of mortality or decreased average daily gain after influenza infection (data not shown).

It is important to mention that most of the farms showing respiratory signs in the pig population were visited and sampled during the summer of 2012, which was unusually hot in Argentina. Clinical signs, however, were reported throughout the year. These results differ from the seasonal pattern reported in the North Hemisphere.[1] This could be explained by: a) intermittent reinfection with antigenically distinct strains; b) the control of the ambient environment applied in the intensive management farms analyzed; and c) the continuous presence of naïve pigs.

The overall seroprevalence of all age groups was of 48·5%. However, when sows and fatteners were analyzed, the prevalence increased to 66·4% and 65%, respectively. Previous studies carried out in Argentina reported lower percentages of positive pigs[14]. In our study, all farms were seropositive to influenza A, although within-farm seroprevalence varied from 7·1 to 79·35%. These results are similar to those of a recent study in Spain using an ELISA test where antibodies against IAV were detected in 93·9% of the farms evaluated.[3] In England, a national study detected antibodies to IAV in 52% of the farms analyzed.[13] Both studies informed a within-herd seroprevalence that ranged from 4 to 100%.[3,13] Differences among studies could be associated with the antigen and test used, the transmission rate of the virus, the farm characteristics (one site or multiple site system, biosecurity, pig flow, replacement policies) or the dissemination of the IAV infection in swine farms after the 2009 pandemic, as suggested by the HI results.[3,14]

In the farms analyzed, two different patterns of infection were observed. In the first one, the low percentage of seropositive pigs in the fattening period suggests active circulation only in the breeding stock, probably caused by an ancient infection. On the contrary, the other pattern shows a clear seroconversion in the post-weaning period in concordance with the decrease in the maternal immunity and an active viral circulation.

In the present study, the antigens used for HI were from strains previously isolated in Argentina[5–7], and the results obtained could be considered representative of the subtypes circulating in pigs in Argentina. Because vaccines to IAV are not licensed in Argentina, these results reflect the situation of IAV infection in non-vaccinated herds. The HI results showed that infection with viruses containing HA's from different subtypes (pH1, δ1H1, δ2H1, and H3) is present among the farms studied (Figure 2). However, as reported elsewhere, the frequency of detection of antibodies against each strain varies[3, 14, 16, 17]. In addition, and in agreement with that reported in several parts of the world, almost 80% of the sera analyzed had antibodies against two or more strains.[3,13,15–17] The HI

results indicated the cocirculation of different subtypes of IAV in the farms, which could lead to reassortment events.[3,17] This has also been reported in Argentina, where two independent reassortant viruses emerged from the combination of pH1N1 internal genes and the surface genes from δ2H1N1 and δ1H1N2 swine influenza viruses.[7]

The GMT was different among farms and categories evaluated. In sows, GMT values were higher than those observed in 160-day-old pigs. The lack of homogeneity of immunity in the categories evaluated warrants the continuous presence of susceptible pigs in the farms. This situation favors the sustainability of the infection in the farms and explains the results observed.[2,3,14,18]

The detection of IAV from nasal swabs of clinical healthy pigs in six of the nine farms studied indicates that this procedure is a useful tool in epidemiological active surveillance, as used in other species.[19] Moreover, in agreement with other studies[4,16,17], a higher detection rate from pneumonic lung lesions was observed. This finding indicates that the viral isolation from lungs with pneumonic lesions could be a better sample than nasal swabs to detect and/or isolate influenza virus in epidemiological studies.

In this study, most of the isolated viruses were pH1. Furthermore, the reassortant subtype of H3N2 of human origin containing pandemic internal genes was isolated. The farm of origin of this reassortant virus had a history of influenza infection with a wholly human H3N2 subtype.[6] This finding suggests that the pH1 has become endemic and that its internal genes are maintained in the pig population by genetic reassortment. The positive selection of the HA and NA genes of pH1 and the concomitant better adaptation to the swine host could be one of the reasons that explains that this subtype is considered the most prevalent IAV subtype in several parts of the world as well as in Argentina.[9,21]

Evidence of IAV lesions was observed in the bronchioli in eight cases. IAV was isolated in four of them, and immunohistochemical studies revealed only one positive case. This result could be attributed to the fact that pigs examined postmortem were those found dead during the day of visit and to the fact that no clinically selected pigs were analyzed or to the low load of virus in the airways, particularly at the level of the bronchioli, where the virus initially multiplied.

This study provides more information about the circulation of IAV and its characteristics in a poorly surveyed region. This study also provides further data that may be used to evaluate the tools necessary to control this disease and thereby improve both the health status of the pig population and the general public health as this is a zoonotic disease.

Addendum

J. Cappuccio, A. Pereda, C. Perfumo, and D. R. Perez contributed to the concept and design of the study; M. Dibárbora and V. Olivera analyzed and interpreted the virological and molecular data; J. Cappuccio, M. Quiroga, and M. Machuca performed necropsies, analyzed, and interpreted the histopathological data; M. Dibárbora, J. Cappuccio, A. Pereda, C. Perfumo, and D. R. Perez revised and approved the final version of the manuscript.

Conflict of interest

The authors declares no conflict of interest

References

1 Olsen CW, Brown IH, Easterday BC et al. Swine influenza. In: Straw B, Zimmerman JJ, D'Allaire S et al. (Ed): Diseases of Swine 9th ed. Ames (IA): Blackwell Publishing; 2006. 469–82.

2 Kyriakis CS, Brown IH, Foni E et al. Virological surveillance and preliminary antigenic characterization of influenza viruses in pigs in five European countries from 2006 to 2008. Zoonoses Public Health 2011; 58:93–101.

3 Simon-Grife M, Martin-Valls GE, Vilar MJ et al. Seroprevalence and risk factors of swine influenza in Spain. Vet Microbiol 2011; 149:56–63.

4 Williamson SM, Tucker AW, McCrone IS et al. Descriptive clinical and epidemiological characteristics of influenza A H1N1 2009 virus infections in pigs in England. Vet Rec 2012; 171:271.

5 Pereda A, Cappuccio J, Quiroga MA et al. Pandemic (H1N1) 2009 outbreak on pig farm, Argentina. Emerg Infect Dis 2010; 16:304–307.

6 Cappuccio JA, Pena L, Dibarbora M et al. Outbreak of swine influenza in Argentina reveals a non-contemporary human H3N2 virus highly transmissible among pigs. J Gen Virol 2011; 92:2871–2878.

7 Pereda A, Rimondi A, Cappuccio J et al. Evidence of reassortment of pandemic H1N1 influenza virus in swine in Argentina: are we facing the expansion of potential epicenters of influenza emergence? Influenza Other Respi Viruses 2011; 5:409–412.

8 Cappuccio JA, Dibárbora M, Barrales H et al. Estudios serológicos y virológicos del virus de influenza A en granjas porcinas pre implementación de planes de vacunación. In: Carranza A, Gabossi H, eds. Procecddings of the XI National Congress of Swine Production. Salta, Argentina: Rio Cuarto, Cordoba, 2012:186.

9   World Health Organization. WHO (2008). Swine Influenza. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2.8.8. Paris: OIE. Available at www.oie.int/Eng/Normes/Mmanual/2008/pdf/2.08.08_SWINE_INFLUENZA. (Accessed 10 February 2013)

10 Spackman E, Senne DA, Myers TJ *et al.* Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol 2002; 40:3256–3260.

11 Hoffmann E, Stech J, Guan Y *et al.* Universal primer set for the full-length amplification of all Influenza A viruses. Arch Virol 2001; 146:2275–2289.

12 Piñeyro PE, Baumeister E, Cappuccio JA *et al.* Seroprevalence of the swine influenza virus in fattening pigs in Argentina in the 2002 season: evaluation by hemagglutination-inhibition and ELISA tests. Rev Argent Microbiol 2010; 42:98–101.

13 Mastin A, Alarcon P, Pfeiffer D *et al.* Prevalence and risk factors for swine influenza virus infection in the English pig population. PLoS Curr 2011; 3:RRN1209.

14 Lopez-Soria S, Maldonado J, Riera P *et al.* Selected Swine viral pathogens in indoor pigs in Spain. Seroprevalence and farm-level characteristics. Transbound Emerg Dis 2010; 57:171–179.

15 Liu W, Wei MT, Tong Y *et al.* Seroprevalence and genetic characteristics of five subtypes of influenza A viruses in the Chinese pig population: a pooled data analysis. Vet J 2011; 187:200–206.

16 Maldonado J, Van Reeth K, Riera P *et al.* Evidence of the concurrent circulation of H1N2, H1N1 and H3N2 influenza A viruses in densely populated pig areas in Spain. Vet J 2006; 172:377–381.

17 Pascua PN, Song MS, Lee JH *et al.* Seroprevalence and genetic evolutions of swine influenza viruses under vaccination pressure in Korean swine herds. Virus Res 2008; 138:43–49.

18 Loeffen WL, Heinen PP, Bianchi AT *et al.* 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 2003; 92:23–35.

19 Fereidouni SR, Harder TC, Gaidet N *et al.* Saving resources: avian influenza surveillance using pooled swab samples and reduced reaction volumes in real-time RT-PCR. J Virol Methods 2012; 186:119–125.

20 Hiromoto Y, Parchariyanon S, Ketusing N *et al.* Isolation of the pandemic (H1N1) 2009 virus and its reassortant with an H3N2 swine influenza virus from healthy weaning pigs in Thailand in 2011. Virus Res 2012; 169:175–181.

21 Li W, Shi W, Qiao H *et al.* Positive selection on hemagglutinin and neuraminidase genes of H1N1 influenza viruses. Virol J 2011; 8:183.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic trees of MP (S1), H1 (S2), H3 (S3) and NA (S4) genes.