Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Epidemiological Analysis of Influenza A Infection in Cambodian Pigs and Recommendations for Surveillance Strategies

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Keywords:
influenza viruses; pigs; risk factors; seroprevalence; surveillance; Cambodia

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Received for publication April 28, 2013

doi:10.1111/tbed.12204

Summary

This study analysed the available data of seroprevalence to human influenza viruses in pigs in Cambodia using generalized linear mixed models in order to improve understanding of factors underlying the spread of human influenza viruses in Cambodian pigs. The associations between seroprevalence against seasonal H1N1 influenza virus in pigs and the population density of humans and pigs were not significant. However, a positive association between anti-H3 antibodies in pigs and the human population density was identified. In contrast, there was a negative association between seroprevalence of H3N2 in pigs and the pig population density. Our study has highlighted the difficulty in identifying epidemiological risk factors when a limited data set is used for analyses. We therefore provide recommendations on data collection for future epidemiological analyses that could be improved by collecting metadata related to the animals sampled. In addition, serosurveillance for influenza A viruses in pigs in high-risk areas or at slaughterhouses is recommended in resource-limited countries.

Introduction

Influenza A viruses are members of the family Orthomyxoviridae and are categorized into different subtypes on the basis of the antigenic properties of envelope glycoproteins, including haemagglutinin (HA) and neuraminidase (NA). Pigs have been proposed to play an important role in the ecology of influenza A viruses due to their susceptibility to influenza viruses from both human and avian species (Brockwell-Staats et al., 2009), facilitating genetic reassortment between viruses and avian-to-human virus adaptation (Ito et al., 1998). These processes possibly lead to the generation of new variants of influenza viruses with pandemic potential (Landolt and Olsen, 2007).

Since its emergence in humans in 2009, the A(H1N1) pdm09 virus has been evolving within pig populations in many countries through reassortment events with other endemic swine influenza strains (Vijaykrishna et al., 2010; Ducatez et al., 2011; Howard et al., 2011; Kitikoon et al., 2011; Moreno et al., 2011; Starick et al., 2011; Tremblay et al., 2011; Fan et al., 2012; Hiromoto et al., 2012). In the USA, the new reassortant of swine H3N2 virus with the M gene from the A(H1N1)pdm09 virus (A(H3N2)v) emerged in pigs in 2009 (Wong et al., 2012). Later in August 2011, the first infection of humans in the USA with the A(H3N2) v virus was reported. It was suspected that the M gene may have contributed to increased transmissibility from pigs to humans and also between people (Wong et al., 2012). The

evidence of novel reassortant viruses emerging in pigs highlights the increasing complexity of influenza virus characteristics that could potentially lead to the generation of new viruses with increased virulence and cross-species transmissibility. Therefore, systematic global surveillance for influenza A viruses in pig populations should be carried out in order to understand the current situation of influenza in the world and to promptly detect the emergence of new influenza variants.

In Cambodia, the livestock sector is dominated by smallholders (Huynh et al., 2007). Poultry and livestock production plays an important role in poverty reduction as well as in wealth creation for smallholders, accounting for nearly 5% of Cambodia's gross domestic product (GDP) and 15.8% of agricultural GDP (Chetra and Bourn, 2009; Tornimbene and Drew, 2012). Primarily, pig management in Cambodia is traditional, where animals are raised with low biosecurity, facilitating the opportunity for contact between humans and pigs. Only a few commercial pig farms exist in Cambodia, and these are located in Kandal Province near Phnom Penh City where they produce pork and other pig products to meet the high urban demand (Chetra and Bourn, 2009; Tornimbene and Drew, 2012). To date, there have been no influenza viruses isolated from Cambodian pigs. However, the recent serological study by Rith et al. (2013) has demonstrated extensive infections with human-origin influenza viruses, including the seasonal H1N1, H3N2 and A(H1N1)pdm09 viruses in pigs in Cambodia. Importantly, evidence that some pigs have been exposed to more than one human influenza virus has been found (Rith et al., 2013). The finding of potential multiple infections with human influenza viruses in pigs represents a substantial risk for pandemic virus creation through reassortment events. Therefore, further studies on human influenza viruses in pigs in Cambodia are essential in order to investigate the factors with a potential to influence disease transmission.

In this study, the only serological data available in Cambodia were used to run generalized linear mixed models in order to study the relationship between infections of seasonal H1N1 and H3N2 influenza viruses in Cambodian pigs and certain environmental factors, including human and pig density. We also provide proposed strategies for enhancing data collection and recommendations for surveillance of influenza viruses in pigs that can help epidemiologists design future studies and surveillance schemes, particularly for low-income countries.

Materials and Methods

Study population

Existing laboratory data (n = 1147) on the serological evidence of infection of pigs from Cambodia with human influenza were examined. The data were sourced from the National Veterinary Research Institute (NaVRI) of Cambodia and the Institut Pasteur in Cambodia (IPC) and represented samples collected between 2006 and 2010. The samples from NaVRI were collected at farms in several provinces, while those from the IPC were obtained from the slaughterhouse in Phnom Penh. The samples were subdivided by sampling occasion based on the province of pig origin, the sampling location and the sampling date (Fig. 1).

Each sample was tested by haemagglutination inhibition (HI) assays for antibodies against the reference strain of seasonal human H1N1 and H3N2 influenza viruses from the year of sampling of that specific sample (Rith et al., 2013). The specimens were considered positive if their HI titres were $\geq$1 : 40. The 95% confidence intervals for the seroprevalence by sampling occasion were calculated using the exact binomial method (Ross, 2003).

The factors that might increase the possibility of human-to-pig contact or facilitate airborne and mechanical transmission of human influenza viruses to pigs were selected. These included the population density of pigs, humans and poultry and road density. The last three factors represented risk factors for highly pathogenic avian influenza (HPAI) H5N1 in several spatial studies (Gilbert et al., 2008; Tiensin et al., 2009; Loth et al., 2010; Yupiana et al., 2010; Martin et al., 2011). The data on pig and poultry population density by province during 2006–2008 were obtained from the report by Chetra and Bourn (2009) (see the Fig S1). Data on human population density by province in 2008 were provided by the National Institute of Statistics, Ministry of Planning, Phnom Penh, Cambodia. The georeferenced road density data were obtained from FAO GeoNetwork (http://www.fao.org/geonetwork/). Data were analysed using ArcGIS9 (Environmental System Research Institute, Redlands, CA, USA).

Statistical analyses

The relationship between seroprevalence to seasonal H1N1 and H3N2 influenza viruses and the selected factors was examined using generalized linear mixed models (GLMMs). Firstly, the variables to include in the model were selected as fixed effects based on Pearson's correlation coefficient (PCC) of each pair of all those factors. According to the study by Graham (2003), pairs of variables with a PCC $\geq$ 0.28 were considered associated and were tested separately in the models. In order to take into account the potential variations between the sources of samples, sampling occasion and year of sampling were included into the models as random effects. All statistical analyses were performed in R (http://www.r-project.org). The models were performed with the 'glmer' function in the 'lme4' package

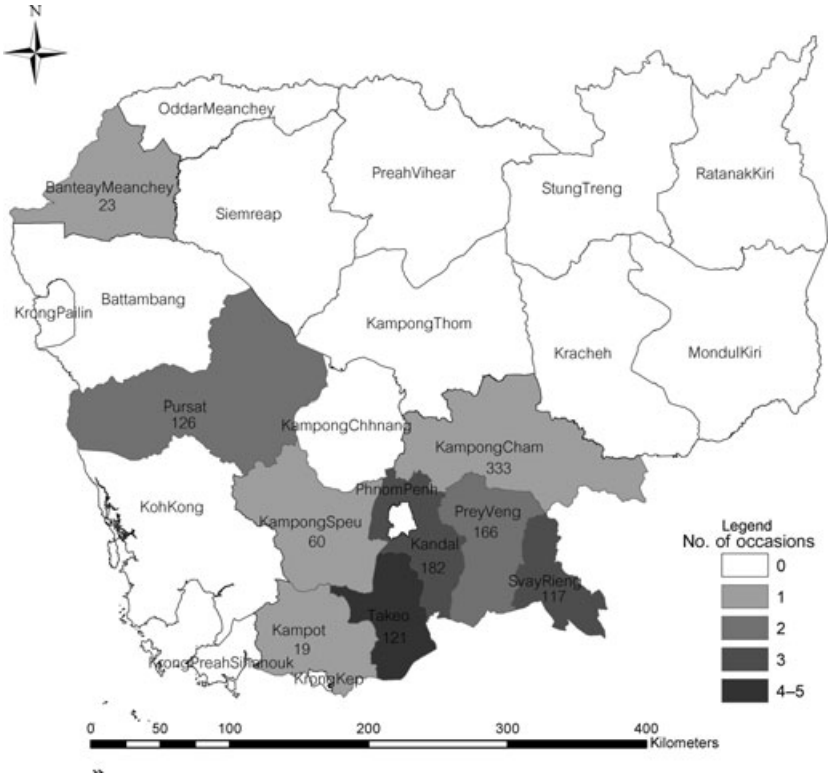

Fig. 1. Map of Cambodia showing administrative units divided into 24 provinces with geographical variations in number of sampling occasions (legend) and number of samples collected in various provinces (number below province name).

in the R environment, using a logit link function with Laplace approximation of a maximum-likelihood method assuming a binomial distribution (Gaidet et al., 2012). The Akaike information criterion (AIC) was used to compare the models.

Results

Seroprevalence by sampling occasion

The seroprevalence against seasonal H1N1 and H3N2 influenza viruses for each sampling occasion is summarized in Table 1. There were 19 sampling occasions from 9 provinces across Cambodia during 2006–2010. The number of serum specimens collected at each sampling occasion varied widely (9–333). The seroprevalence against seasonal H1N1 virus ranged from 0 to 52.2%, whereas the prevalence of anti-H3 antibodies varied from 0 to 43.3%.

Selection of fixed effects

Road density and poultry density were removed from the model because of their high correlation coefficient with human density and pig density, respectively (Table 2). The two variables included in the model as fixed effects were human density and pig density because we expected that these two variables would have the strongest impact on the pig seroprevalence, despite their PCC being superior to 0.28 (Table 2).

The models used for investigating factors influencing seroprevalence

According to AIC, the high-ranking models fitted to analyse the variations in seroprevalence against seasonal H1N1 and H3N2 influenza viruses in Cambodian pigs are shown in Tables 3 and 4, respectively. The best-supported models for H1N1 and the top three best-supported models for H3N2 included human population density and pig population density (Tables 3 and 4). The random effects of year and sampling occasion contributed to most of the random part of the high-ranking models. The highest ranking model for H1N1 included sampling occasion as a random effect, while that for H3N2 showed year mostly accounted for the random part (Tables 3 and 4).

Statistical analyses

No significant association between seroprevalence of H1N1 and the population density of humans or pigs was found (Table 3). Seroprevalence of H3N2 was positively

Table 1. Results of seroprevalence of seasonal H1N1 and H3N2 antibodies by sampling occasion and data on investigated factors for each sampling occasion used in the statistical analyses

Occasion							% Seroprevalence (95% CI)		Human density	Pig density	Poultry density	Road density
no.	Province	Month	Year	Source	Location	*n*	H1N1 subtype	H3N2 subtype	(persons per km^2)	(no. per km^2)	(no. per km^2)	(length per km^2)
1	Banteay Meanchey	5, 6, 7, 8	2008	IPC	Abattoir	23	52.2 (30.6–73.2)	13.0 (2.8 –33.6)	101.5	16.3	78.1	9.5
2	Kampong Cham	2	2006	NaVRI	Farm	333	4.2 (2.3 –7.0)	2.7 (1.2 –5.1)	171.5	21.2	217.1	8.9
3	Kampong Speu	7	2006	NaVRI	Farm	60	15.0 (7.1 –26.6)	0.0 (0.0 –6.0)	102.1	23.7	206.5	7.8
4	Kampot	12	2007	IPC	Abattoir	19	0.0 (0.0 –17.6)	0.0 (0.0 –17.6)	120.1	29.9	226.9	9.6
5	Kandal	12	2007	IPC	Abattoir	25	12.0 (2.5 –31.2)	0.0 (0.0 –13.7)	354.6	38.9	326.5	16.5
6	Kandal	6, 7, 8, 9	2008	IPC	Abattoir	97	53.6 (43.2 –63.8)	43.3 (33.3 –53.7)	354.6	32.4	281.5	16.5
7	Kandal	11	2010	IPC	Abattoir	60	8.3 (2.8 –18.4)	3.3 (0.4 –11.5)	354.6	32.4	281.5	16.5
8	Prey Veng	12	2007	IPC	Abattoir	9	0.0 (0.0 –33.6)	0.0 (0.0 –33.6)	194.0	82.7	421.3	9.2
9	Prey Veng	11	2010	IPC	Abattoir	157	12.7 (8.0 –19.0)	2.6 (0.7 –6.4)	194.0	69.9	442.3	9.2
10	Pursat	12	2007	IPC	Abattoir	27	0.0 (0.0 –12.8)	0.0 (0.0 –12.8)	31.3	6.5	77.8	5.9
11	Pursat	5, 6, 7, 8, 9	2008	IPC	Abattoir	99	46.5 (36.4 –56.8)	42.4 (32.5 –52.8)	31.3	6.4	67.7	5.9
12	Svay Rieng	12	2007	IPC	Abattoir	12	0.0 (0.0 –26.5)	0.0 (0.0 –26.5)	162.8	59.6	268.5	13.4
13	Svay Rieng	5, 6, 7, 8	2008	IPC	Abattoir	50	30.0 (17.9 –44.6)	8.0 (2.2 –19.2)	162.8	59.6	268.5	13.4
14	Svay Rieng	11	2010	IPC	Abattoir	55	16.4 (7.8 –28.8)	3.6 (0.4 –12.5)	162.8	59.6	268.5	13.4
15	Takeo	12	2007	IPC	Abattoir	21	0.0 (0.0 –16.1)	0.0 (0.0 –16.1)	236.9	58.9	520.8	10.8
16	Takeo	11	2009	NaVRI	Farm	36	13.9 (4.7 –29.5)	19.4 (8.2 –36.0)	236.9	51.4	627.9	10.8
17	Takeo	8, 11	2010	NaVRI	Farm	25	0.0 (0.0 –13.7)	0.0 (0.0 –13.7)	236.9	51.4	627.9	10.8
18	Takeo	2	2010	NaVRI	Farm	11	18.2 (2.3 –51.8)	0.0 (0.0 –28.5)	236.9	51.4	627.9	10.8
19	Takeo	11	2010	IPC	Abattoir	28	21.4 (8.3 –41.0)	0.0 (0.0 –12.3)	236.9	51.4	627.9	10.8

Table 2. The Pearson correlation coefficients calculated for selecting fixed effects in the models

Variables	Human density	Pig density	Poultry density	Road density
Human density	1.0			
Pig density	0.38	1.0		
Poultry density	0.54	0.67	1.0	
Road density	0.84	0.31	0.20	1.0

associated with density of the human population, whereas it was negatively related to the pig population density (Table 4).

Discussion

Seroprevalence data on seasonal human H1N1 as well as seasonal human H3N2 influenza viruses in Cambodian pigs used in this study were obtained from a study previously reported by Rith et al. (2013). All serum specimens were tested by HI assays, which are the prime serological test for influenza A viruses in pigs (Van Reeth et al., 2006). It is important to note that serological cross-reactivity between subtypes could be observed if pigs on farms had been infected or vaccinated with various influenza virus subtypes and variants, including swine influenza viruses (Van Reeth et al., 2006). Recently, serological cross-reaction to the A (H1N1)pdm09 virus has been detected in pigs that were previously infected or vaccinated with European swine influenza viruses (Kyriakis et al., 2010). To our knowledge however, no autogenous or commercial vaccines against swine influenza have been used in Cambodian pigs. The respiratory specimens of 1000 pigs sampled in a slaughterhouse in Phnom Penh during 2006–2008 all tested negative for influenza A viruses using molecular techniques, probably because farmers refrained from sending sick animals to the slaughterhouse as they would be examined by veterinarians and rejected if symptoms were observed (Institut Pasteur in Cambodia, unpublished data). In addition, antibodies against A(H1N1)pdm09 were not detected in pigs before the virus started to widely circulate in the human population in Cambodia (Rith et al., 2013), suggesting that swine influenza viruses capable of generating cross-reactive antibodies against the A(H1N1)pdm09 virus did not previously circulate in Cambodian pigs. Thus, we

Table 3. Summary of the best-supported models fitted to estimate the variations in seroprevalence against seasonal H1N1 influenza viruses in pigs in Cambodia

			Random effect[a]		Estimate		Pr(>|z|)	
Model	AIC	*k*	Occasion	Year	Human density	Pig density	Human density	Pig density
1	52.70	3	1.2581		0.0012	−0.0083	0.15	0.14
2	52.78	2	1.2564			−0.0058		0.27
3	52.90	2	1.2804		0.0008		0.30	
4	54.70	4	1.2581	9.27E-13	0.0012	−0.0083	0.15	0.14
5	54.78	3	1.2564	5.78E-17		−0.0058		0.27
6	54.90	3	1.2804	0.00	0.0008		0.30	
7	61.38	2		1.2674		−0.0048		0.37
8	61.61	2		1.2755	0.0006		0.45	
9	62.22	3		1.2556	0.0009	−0.0066	0.28	0.24

[a]Variance estimation.

Table 4. Summary of the best-supported models fitted to estimate the variations in seroprevalence against seasonal H3N2 influenza viruses in pigs in Cambodia

			Random effect[a]		Estimate		Pr(>|z|)[b]	
Model	AIC	*k*	Occasion	Year	Human density	Pig density	Human density	Pig density
1	47.52	3		3.3149	0.0031	−0.0350	0.0030 **	9.03E-05 ***
2	47.68	3	4.8402		0.0030	−0.0345	0.0040 **	9.7E-05 ***
3	49.52	4	1.92E-10	3.3148	0.0031	−0.0350	0.0030 **	9.03E-05 ***
4	54.09	2	5.0361			−0.0217		0.0015 **
5	54.44	2		3.3022		−0.0215		0.0016 **
6	56.09	3	5.0360	1.26E-11		−0.0217		0.0015 **
7	64	2	4.8164		0.0008		0.3740	
8	64.11	2		3.2186	0.0009			0.3415
9	66.01	3	1.2590	2.6569	0.0008		0.3642	

[a]Variance estimation.
[b]Significant codes: 0 '***', 0.001 '**'.

speculate that the serological results demonstrating infection with influenza viruses of human origin in Cambodian pigs most likely reflected the real situation.

The associations between seroprevalence to seasonal H1N1 and H3N2 influenza viruses in pigs and two selected factors, human density and pig density, were determined. No associations between seasonal influenza prevalence to H1N1 in pigs and the studied factors were detected. The seroprevalence against H1N1 could be complicated by an appearance of the A(H1N1)pdm09 virus after its emergence in April 2009 as the virus has replaced the seasonal influenza A(H1N1) virus in humans worldwide and also has been circulating in pig populations following transmission from humans to pigs (Forgie et al., 2011). The seroprevalence of H3N2 influenza virus in pigs was positively associated with the density of the human population. This finding suggests that the high density of humans in Cambodia may contribute to the high level of infection in pigs, possibly by spill-over from humans through close contact between humans and pigs on farms. Road density was not included in the model because it was highly correlated with human density. Thus, the correlation between human density and H3N2 seroprevalence may also be related to a high road density that could have resulted from the transportation of infected pigs and contaminated fomites, which may play a key role in human-mediated transmission of influenza viruses to pigs. Overall, these findings indicate that infections of Cambodian pigs with human influenza viruses could be related, in general, to human activities.

The seroprevalence of H3N2 influenza virus in Cambodian pigs was negatively associated with the density of the pig population, suggesting that provinces with high pig density have fewer influenza infections. This may imply

that pigs in the areas with high population densities are bred in commercial farms with good husbandry practices. Indeed, <1 per cent of pig producers operate on a commercial level (Huynh et al., 2007; Tornimbene and Drew, 2012). Thus, this result should be interpreted with caution because the statistical analysis may be compromised by some level of spatial bias that could have arisen from the non-randomly collected samples. To illustrate, the data analysed represented combined serological results of serum samples that were sourced from the NaVRI's surveys at farms in 3 provinces and the IPC's surveys at the slaughterhouse in Phnom Penh. Moreover, data on the density of the pig population were analysed at a provincial level, which may cover different densities of pig population. The underlying variations in pig farm management within the provinces, such as husbandry practices, biosecurity and hygiene, may also interfere with the result. Therefore, this study has demonstrated the limitations of analysing risk factors when relying on pre-existing surveys with limited data sets.

Although the data were useful to assess the circulation of the virus or to determine the dominant influenza strains in pigs in the country (Rith et al., 2013), using them to identify risk factors appears to be challenging owing to the data set not being comprehensively distributed and the limited supporting information (Fig. S1) of the data set collected. In the absence of accurate spatial data, the only metadata available here were the origin of the pigs at a provincial level, making our analysis restricted to a provincial scale. Thus, the results from this study can only be used to crudely analyse the association between the prevalence of human influenza viruses in pigs and certain factors. We recommend that sampling methods should be improved and metadata related to the animals sampled should be systematically collected at farms or abattoirs for epidemiological studies (prevalence, case–control studies, etc.) in order to effectively identify and quantify the drivers of influenza virus infection in pigs. The list of essential data should include precise farm locations (at least the village and district of origin of the animal), animal age, farm type and farm husbandry practices that would provide accurate spatial data of the animal origin and informative data on potential risk factors for epidemiological investigations. Such data would be particularly useful in the case when environmental factors were retrospectively analysed, for example, for risk mapping.

To implement the surveillance for animal diseases, surveillance plans and systems should be designed and adapted to the local socio-economic context. In low-income countries, where financial and technical resources are finite, it would be impossible to conduct nationwide surveillance. Thus, surveillance efforts in the context of weak infrastructure should be targeted in order to maximize cost-effectiveness. Developing a targeted surveillance within the concept of risk-based surveillance could be accomplished by focusing on subpopulations that are anticipated to have a higher risk of disease infections so as to utilize fewer resources resulting from the smaller sample size required. Places such as markets and slaughterhouses, where pigs from numerous origins commingle, would be appropriate for sample collection because these places facilitate the concentration and dissemination of infectious agents from various geographical origins and also provide a large source of biological samples that can be collected at one time.

Given increasing global awareness of potential pandemics, the main objective of surveillance for influenza A viruses in pigs is to detect new strains of influenza viruses early. These can be carried out directly by virus detection or detecting its genetic material and also indirectly by detecting antibodies (Torremorell et al., 2012) or using relevant 'markers' through non-specific (syndromic) surveillance. Virological monitoring by means of active surveillance is usually recommended for studying the genetic components of influenza viruses because the continued circulation of influenza viruses in pigs has raised concerns about the risk for genetic evolution and potential re-transmission of new variants back to humans with increased pathogenicity (Liu et al., 2012; Trevennec et al., 2012). However, due to the low isolation rates of influenza viruses from pigs in East and South-East Asia (Trevennec et al., 2011), a large number of biological samples would need to be collected in order to successfully detect the viruses. The practical use of virological monitoring is therefore impeded by its high cost, time consumption, high technical demand for laboratory capacity and a need for skilled workers.

Two main approaches of influenza surveillance are suggested. Firstly, at-risk farms that are epidemiologically linked to cases of influenza-like illness in pigs or humans should be targeted. Importantly, specimens should be obtained from both healthy and clinically ill pigs at those farms as subclinical infections can occur. To promptly detect farms with animals showing respiratory signs, a good passive network and education campaign are required in order to encourage farmers to report respiratory cases that could efficiently be organized through a sentinel network of farms in a high-risk area. Secondly, surveillance should be based on the assessment of antibody levels. Although serological data are often thought to be of limited value as it could be complicated by maternal antibodies, serologic cross-reactivity, endemic disease and vaccination status in the country, the testing is rapid, relatively inexpensive and easy to perform. Moreover, antibodies could be detectable for several weeks so that the chance of detecting antibodies is higher than that of

detecting virus, which is limited by the short period of virus shedding (Torremorell et al., 2012). In resource-limited countries, serological testing for disease surveillance is therefore appropriate.

Seromonitoring could be used to indicate long-term trends and to detect disease emergence by computing a baseline of seroprevalence in defined pig populations. In particular, systematic sample collection from pigs at abattoirs would provide a good spatiotemporal picture of circulating subtypes, because the pigs presented are usually older than those on farms and consequently have more opportunity to have been exposed to or infected with influenza viruses. Defining a baseline level of antibodies may help the early detection of influenza outbreaks in target pig populations by recognizing an increased seroprevalence. The use of a large panel of antigens for HI assays is recommended to correctly identify the dominant strains in the country and also to rapidly detect any unusual subtypes of influenza A viruses in pig populations. Furthermore, antigens for HI tests should be regularly compared with those from circulating strains in a given country or region (Sreta et al., 2013).

Our study demonstrated an epidemiological analysis of influenza A virus transmission in Cambodian pigs that shows a positive association with the human population density and a negative relationship with pig population density. These unexpected results may be linked to poor data collection and sampling strategy that could interfere with the statistical analysis. The need for improved data collection and surveillance schemes in Cambodia or other countries where funds are limited has thus been emphasized. To further refine these surveillance strategies, a better understanding of human–pig–avian interfaces and detailed pig trade in the country are needed. Moreover, socio-economic studies are required to adapt the surveillance schemes to the local context.

Acknowledgements

This work was supported by the GREASE Network (http://grease-network.com) and by the Office of the Assistant Secretary for Preparedness and Response within the U.S. Department of Health and Human Services.

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Supporting Information

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

Figure S1. Poultry and pig population density by Cambodian Province in 2006–2008.