The Epidemiology of influenza A viruses in pigs in Thailand and Cambodia

By

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Declaration

I declare that this thesis is a true account of my research and contains as its main content work that was conducted by me, and that the work has not previously been submitted for a degree at any other tertiary education institution.

Punnaporn Netrabukkana
Abstract

In April 2009, a novel influenza H1N1 virus (A(H1N1)pdm09 virus) emerged in humans in California and Mexico and quickly spread worldwide through human-to-human transmission, resulting in the World Health Organization declaring a phase 6 pandemic. The virus was found to be a swine–human–avian triple reassortant virus, with a unique combination of gene segments derived from North American and Eurasian swine lineages. Soon after its detection in humans, the A(H1N1)pdm09 virus was isolated from pigs around the world. Transmission of influenza A viruses between humans and pigs is of public health concern because reassortment can take place in the pig reservoir and further cross-species infections may facilitate creation of a new reassortant influenza virus with pandemic potential.

The first study in rural Thailand demonstrated that farmers’ and traders’ practices may constitute substantial risks for interspecies influenza virus transmission, thereby posing a threat to pig populations and public health. A cross-sectional knowledge, attitude and practices (KAPs) survey of pig smallholders showed that most farmers had limited knowledge of influenza A in pigs. This has significant public health implications and will be important when developing influenza prevention and control programs in the future. Virus isolation was attempted from a number of small and commercial pig farms in Thailand. No positive samples were recovered from pigs on small farms, whereas 8 isolates of influenza A virus were isolated from weaning pigs from commercial farms. Two viruses isolated in the study were found to be reassortant H3N2/A(H1N1)pdm09 viruses. The other six influenza A viruses isolated were all the A(H1N1)pdm09 virus strain. Virological surveillance for
influenza A viruses in the Thai pig population should be continued to study virus evolution and improve the understanding of virus ecology to benefit the pig industry and public health.

Results from a study in Cambodia indicated sustained transmission of human influenza virus infections in pigs. Antibodies against seasonal H1, H3 and A(H1N1)pdm09 subtypes were common in Cambodian pigs. Despite numerous outbreaks in chickens in Cambodia, infection with the H5 subtype related to avian influenza was not detected in pigs. The historical seroprevalence data for human influenza viruses in Cambodian pigs were further analysed. Associations between seroprevalence against seasonal H1N1 influenza virus in pigs and the population densities of humans and pigs were not significant. However a positive association was found between anti-H3 antibodies in pigs and the human population density. In contrast, there was a negative association between seroprevalence of H3N2 in pigs and the pig population density. These findings highlighted the difficulty in identifying epidemiological risk factors when a limited dataset is used for analyses and thus recommendations on data collection for future epidemiological analyses were provided.
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<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>AIC</td>
<td>Akaike Information Criterion</td>
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<td>AIVs</td>
<td>Avian Influenza Viruses</td>
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<td>CDC</td>
<td>United States Center for Disease Control and Prevention</td>
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<td>CPE</td>
<td>Cytopathic Effects</td>
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<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<td>EVAP</td>
<td>Evaporative Cooling System</td>
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<tr>
<td>GDP</td>
<td>Gross Domestic Product</td>
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<td>GLMMs</td>
<td>Generalized Linear Mixed Models</td>
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<tr>
<td>HA</td>
<td>Haemagglutinin</td>
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<tr>
<td>HI</td>
<td>Haemagglutination Inhibition</td>
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<tr>
<td>HPAI</td>
<td>Highly Pathogenic Avian Influenza</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IPC</td>
<td>Institut Pasteur in Cambodia</td>
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<tr>
<td>KAPs</td>
<td>Knowledge, Attitudes and Practices</td>
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<tr>
<td>M</td>
<td>Matrix</td>
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<td>MDCK</td>
<td>Madin–Darby Canine Kidney</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MN</td>
<td>Microneutralisation</td>
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<tr>
<td>NA</td>
<td>Neuraminidase</td>
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<tr>
<td>NaVRI</td>
<td>National Veterinary Research Institute</td>
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<tr>
<td>NIAH</td>
<td>National Institute of Animal Health</td>
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<tr>
<td>NP</td>
<td>Nucleoprotein</td>
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<tr>
<td>NS</td>
<td>Nonstructural Proteins</td>
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<tr>
<td>OIE</td>
<td>World Organisation of Animal Health</td>
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<tr>
<td>PA</td>
<td>Polymerase Acidic</td>
</tr>
<tr>
<td>PB</td>
<td>Polymerase Basic</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PCC</td>
<td>Pearson’s Correlation Coefficient</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PNP</td>
<td>Proliferative and Necrotizing Pneumonia</td>
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<tr>
<td>PRDC</td>
<td>Porcine Respiratory Disease Complex</td>
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<tr>
<td>PRRS</td>
<td>Porcine Reproductive and Respiratory Syndrome</td>
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<tr>
<td>PSP-D</td>
<td>Porcine Surfactant Protein D</td>
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<tr>
<td>RBCs</td>
<td>Red Blood Cells</td>
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<td>RDE</td>
<td>Receptor-Destroying Enzyme</td>
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<td>RNP</td>
<td>Ribonucleoprotein</td>
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<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription-Polymerase Chain Reaction</td>
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<td>SA</td>
<td>Sialic Acid</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>SI</td>
<td>Swine Influenza</td>
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<tr>
<td>SIV</td>
<td>Swine Influenza Virus</td>
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<tr>
<td>SIVs</td>
<td>Swine Influenza Viruses</td>
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<tr>
<td>TCID</td>
<td>Tissue Culture Infectious Dose</td>
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<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
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<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
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<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER 1: GENERAL INTRODUCTION

Influenza A viruses are enveloped viruses with segmented negative-strand RNA genomes. Traditionally, influenza A viruses are categorised into different subtypes based on the antigenic properties of envelope glycoproteins comprising haemagglutinin (HA) and neuraminidase (NA) (Ma et al. 2009). Due to the segmented nature of the influenza viral genome, reassortment events can occur when a host is simultaneously infected with 2 distinct subtypes (Webster et al. 1992). This has a significant public health implication as it could potentially lead to the generation of novel reassortant viruses with pandemic potential (Landolt and Olsen 2007).

Swine influenza is among the most prevalent respiratory diseases of pigs and is endemic in most pig populations worldwide (Brown 2000). Most common subtypes of influenza A infections in pigs are H1N1, H3N2 and H1N2 (Webby et al. 2004). Importantly, pigs are considered as ‘mixing vessels’ for genetic reassortment of influenza viruses from different species, owing to their dual susceptibility to influenza viruses of avian and mammalian origins (Ito et al. 1998). Furthermore, the frequent exchange of influenza A viruses between pigs and humans, or pigs and birds, is facilitated directly by regular close contact during routine husbandry and management practices (Brown 2000). Consequently, pigs have often been involved in interspecies transmission and implicated in the emergence of new influenza strains.
In the 2009 influenza pandemic, the molecular evidence revealed that pigs may be implicated in the creation of the causative A(H1N1)pdm09 virus as the virus contained a unique genome constellation derived from swine influenza viruses (SIVs), namely the classical swine H1N1 lineage, the North American H3N2 triple-reassortant and the Eurasian ‘avian-like’ swine H1N1 virus (Garten et al. 2009). After its widespread infections in humans, the A(H1N1)pdm09 virus has become established in pig populations across the world following primary contact with infected humans (Brookes and Brown 2011). Moreover, it 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 particular, the new reassortant of an H3N2 SIV with the M gene from the A(H1N1)pdm09 that emerged in pigs in the USA subsequently infected humans (Wong et al. 2012).

In Thailand, influenza A virus subtypes of H1N1, H3N2 and H1N2 have been found in pig populations for several decades (Takemae et al. 2008). Evidence of virus transmission between pigs and humans has been reported with a swine-like influenza virus being isolated from a Thai patient (Komadina et al. 2007). Pig-to-human influenza virus transmission was also confirmed in Thai pig farm workers (Kitikoon et al. 2011). Conversely, studies by Takemae et al. (2008) and Lekcharoensuk et al. (2010) suggested that the Thai H3N2 SIVs acquired the haemagglutinin gene by the introduction of human H3N2 virus into the Thai pig population. After the emergence of the A(H1N1)pdm09 virus in humans, the virus and its reassortants have continued
to be isolated from Thai pigs (Hiromoto et al. 2012). In Cambodia, on the other hand, there have been no influenza viruses isolated from pigs to date (Rith et al. 2013).

The complexity of influenza A viruses in pigs has emphasised the critical need for a deeper understanding of the epidemiology of these viruses, particularly in Southeast Asia, considered a potential epicenter for the emergence of pandemic viruses (Nelson et al. 2007). In particular, an assessment of knowledge, attitudes, and practices related to influenza A in pigs among high-risk groups is essential in order to refine disease control strategies to make them suitable for adoption in the local context. Thus, this thesis reports on a series of epidemiological studies of influenza A viruses in pigs to increase our knowledge and understanding of the situation in pigs in Thailand and Cambodia. The studies are as follows: a) molecular characterisation of influenza A viruses in pigs from central Thailand; b) assessment of potential risks for influenza A transmission at the pig-human interface on small pig farms in rural Thailand; c) serological and virological surveys for influenza A viruses in pigs in rural Thailand; d) a knowledge, attitudes and practices (KAP) survey of Thai pig smallholders towards influenza in rural Thailand; e) serological surveys for influenza A viruses in pigs in Cambodia; and f) an epidemiological analysis of factors influencing influenza A virus infections in pigs in Cambodia.

I would like to state that I carried out all the work documented in the thesis, working in multidisciplinary research teams who provided me with the opportunities, funds and support to carry out my research, with my results contributing to their larger, longer term studies. To illustrate, for the work on molecular characterisation of
influenza A viruses in pigs from central Thailand (Chapter 3), I was the principal student investigator and in this aspect of my study was supervised by Japanese researchers working on a large influenza study in pigs in Thailand. I conducted the investigations on the farms, gathered farm information, collected samples, conducted the laboratory work and performed the data analyses. Regarding the Mukdahan study detailed in Chapters 4-6, I made a substantial contribution to the overall study design as part of a team of researchers. I undertook the fieldwork, data interpretation and data analysis as well as providing the report that formed the basis of the subsequent publication. For the work described in Chapters 7-8, I participated actively in the execution of the serological study. I collected the samples at the slaughterhouse in Phnom Penh in 2010, conducted the necessary laboratory work at the Institut Pasteur in Cambodia, analysed the data and produced the manuscript for publication.
Chapter 2: Literature review

2.1 Introduction

Influenza viruses are members of the family Orthomyxoviridae, which consists of five genera including Influenza A, B and C viruses, Thogotovirus and Isavirus (Vincent et al. 2008). The influenza A, B and C viruses are characterised by segmented, negative-strand RNA genomes (Bouvier and Palese 2008). Of these, only influenza A viruses are true zoonotic agents. Influenza B and C viruses are primarily human pathogens. Influenza C viruses can occasionally infect pigs and dogs (Ohwada et al. 1987). Influenza A viruses are distinguished from type B and C viruses on the basis of the identity of the major internal protein antigens, namely nucleoprotein (NP) and matrix (M1) protein (Webster et al. 1992). Influenza A viruses are typed according to their surface glycoproteins including HA and NA. The combination of the HA and NA designates the subtype of influenza A viruses. To date, there have been sixteen antigenically different HA (H1-H16) and 9 different NA (N1-N9) recognised (Van Reeth 2007).

2.1.1 Components of the virion

Influenza A viruses are 80–120 nm particles (see Figure 2.1), consisting of a host-derived lipid bilayer envelope, the virus-encoded glycoproteins (HA and NA), the embedded matrix protein (M2), the inner shell of M1 and the nucleocapsids of the viral genome with eight segments of single-stranded RNA at the center (Webster et al. 1992). The RNA fragments are loosely encapsulated by multiple NP molecules.
The trimetric RNA polymerase complexes containing the three viral polymerase proteins (polymerase basic 1 or PB1, PB2 and polymerase acidic or PA) are located at the ends of the nucleocapsids (Webster et al. 1992). The polymerase complex combined with viral RNA and NP is called the ribonucleoprotein (RNP) complex (Vincent et al. 2008).

**Figure 2.1.** Schematic representation of an influenza A virus (Heinen 2003).

The eight viral RNA segments encode 10 recognised gene products. These are PB1, PB2 and PA polymerases, HA, NP, NA, M1 and M2 proteins, and nonstructural NS1 and NS2 proteins. The HA and NA proteins are important for the determination of host range, antigenicity and pathogenicity (Thacker and Janke 2008). Moreover, they
are the main targets of the host humoral immune response and thus serve as targets for diagnostic assays. The HA is the viral receptor-binding protein and mediates fusion of the virus envelope with the host cell membrane (Skehel and Wiley 2000). The NA protein is responsible for cleaving terminal sialic acid residues from carbohydrate moieties on the surfaces of the host cell and the virus (Gottschalk 1957). It aids in virus cell entry by mucus degradation (Matrosovich et al. 2004) and helps release and spread progeny virions (Palese et al. 1974). The M2 protein functions as an ion channel (Wang et al. 1993). The M1 protein is the most abundant protein present in the influenza virion, lying beneath the lipid envelope. The nonstructural proteins (NS1 and NS2) are abundant in infected cells, particularly NS1. The NS1 is primarily in the nucleus and the NS2 is primarily in the cytoplasm, but they are not incorporated into progeny virions. Although both proteins play roles in virus replication, those roles have not been fully defined. The NS2 appears to modulate the synthesis of NS (Webster et al. 1992). Three proteins, including PA, PB1 and PB2, are involved in virus replication. Lastly, the NP protein is a nucleocapsid structural protein (Reid and Taubenberger 2003).

2.1.2 Influenza A virus replication cycle

The influenza A virus replication cycle is illustrated in Figure 2.2. It begins with the cleavage of HA into HA1 and HA2 by enzymes in the respiratory tract. The enzymes are produced by hosts or may be derived from bacteria in order to promote influenza infections (Heinen 2003). An influenza virus particle with cleavage-activated HA binds to cells through interaction between the receptor-binding site of HA and the terminal sialic acid of the cell surface receptor glycoprotein or glycolipid. After attaching to the host cell, the virus particle is endocytosed (Heinen 2003). The low
pH of the endocytotic vesicle triggers a conformational change in cleaved HA, assisting the insertion of the hydrophobic free amino terminus of HA2 into the vesicular membrane and introducing fusion of the viral and vesicular membranes. Finally, the fusion releases the contents of virions into the cytoplasm of the cell (Webster et al. 1992).

After the nucleocapsids of the parent virus migrate into the host cell nucleus, their associated polymerase complexes initiate primary transcription of mRNA. The primary transcripts are used for translation of viral proteins, which are the prevailing NP and NS1 in the early stage of infection. Translation of host mRNAs is blocked. Newly synthesised NP and NS1 migrate to the nucleus. It is believed that the increased concentration of free NP triggers the shift from mRNA synthesis to cRNA and vRNA synthesis by the infection of viral genome. Newly synthesised vRNAs are encapsulated in NP within the nucleus and perform as templates for secondary transcription of viral mRNAs. The predominant translation products in the latter stage of infection are M1, HA and NA proteins. The HA and NA proteins are posttranslationally processed and transported to the cell surface where they integrate into the cell membrane (Webster et al. 1992).

Regarding the assembly process of influenza viruses, a viral core of nucleocapsids becomes encased in a shell of M1 protein and buds outward through the cell membrane. It encloses itself in a bubble of membrane as its own envelope, coated with the viral surface glycoproteins. Interactions between M1 and the cytoplasmic domains of HA, NA or M2 have been proposed as signals for budding. NA releases progeny virions from the host cell (Webster et al. 1992). Subsequently progeny
Virions can infect other cells or can be transmitted to another individual (Heinen 2003).

**Figure 2.2.** Schematic diagram depicting the replication cycle of an influenza virus (Murphy and Webster 1996).

### 2.1.3 Mechanisms of the genetic evolution

The two main mechanisms of evolution of influenza A viruses are: antigenic drift and antigenic shift.
2.1.3.1 Antigenic drift

Antigenic drift refers to the gradual accumulation of point mutations in viral proteins, mostly the HA, in response to immune pressure in populations (Olsen 2002). The number of mutations and the mutation rate vary significantly between viruses, genes and gene regions (Truyen et al. 1995). The lack of proofreading of RNA polymerases could lead to replication errors in the order of 1 in 104 bases (Holland et al. 1982). Each round of RNA virus replication contributes to a mixed population with many variants, most of which are not viable, but some of which have potentially advantageous mutations that can become dominant under the right selective conditions. It has previously been reported that the rate of drift among SIVs was thought to be significantly slower than for human influenza viruses (Webster et al. 1992). Following transmission to pigs, the rate of antigenic drift is lower than in the natural hosts (Brown 2000).

2.1.3.2 Antigenic shift

Antigenic shift is an abrupt genetic and antigenic change, which can create a new subtype of influenza A virus to start circulating in a given population. This results from three mechanisms as follows: 1) genetic reassortment between a virus currently circulating in a population and one or more new viruses (Olsen 2002); 2) the direct transfer of a whole virus from one host species into another and 3) the re-emergence of a virus that was found previously in a species but is no longer in circulation (CFSPH 2009). Reassortment is possible whenever two different influenza viruses infect a cell simultaneously. After co-infection of a cell genome, segments of different strains can be mixed and rearranged in the progeny viruses. Since a single virus particle contains each of the eight unique RNA segments, it is possible to
obtain 256 different possible genotypes from two parental viruses, potentially generating new viruses with very distinct biological properties (Truyen et al. 1995). This happens among influenza A viruses in nature and is essential for the occurrence of pandemics in human populations (Webster et al. 1992).

2.1.4 Molecular determinants of host range restriction

Host range restriction of influenza A viruses is known to be partial because a number of occurrences of interspecies transmission have been reported. The determining factors of host range restriction have not been confirmed. However, the following virus gene products appear to play a significant role as molecular determinants (Webster et al. 1992).

2.1.4.1 Haemagglutinin (HA)

The HA has been considered as a determinant of host range due to its role in host cell recognition and attachment (Webster et al. 1992). Some HA subtypes are restricted to certain mammalian species such as H1, H2 and H3 in humans; H1 and H3 in pigs; and H3 and H7 in horses. One explanation is that some unrecognised features of these subtypes contribute to the peculiarity of adaptation for growth in those mammals or it may be an accident of recent history (Webster et al. 1992).

Previously, it has been demonstrated that subtle changes in the amino acid residues that constitute the binding site can cause alterations of the receptor-binding properties of the HA molecule (Landolt and Olsen 2007). Moreover, the number and position of N-linked oligosaccharides at or around the receptor-binding site contribute to receptor-binding specificity (Deom et al. 1986;
Aytay and Schulze 1991; Gunther et al. 1993). The variation of glycosylation around the receptor-binding site is often seen after adaptation of a virus to growth in a new host species or cell line. The competitive inhibitors may also be associated with host range restriction (Ryan-Poirier and Kawaoka 1991). For example, the studies by Hartshorn et al. (2000) demonstrated that sialic acid (SA) residues on porcine surfactant protein D (PSP-D) may influence host range by acting as natural inhibitors of influenza virus binding to cell-surface SA receptors.

2.1.4.2 Neuraminidase (NA)
Balanced action between HA receptor binding affinity and NA receptor-destroying activity affects efficient growth of influenza virus (Baum and Paulson 1991; Rudneva et al. 1993; Gubareva et al. 1996; McKimm-Breschkin et al. 1996). Differences in the NA’s sialic acid substrate specificity and cleavage activity are often involved in alterations in HA receptor-binding preference (Baum and Paulson 1991; Kaverin et al. 1998). The NA molecule contains a stalk region that is inserted into the viral envelope. Importantly, the length and amino acid sequence of the stalk region vary substantially between different viruses (Blok and Air 1982). Additionally, the growth characteristics of viruses in embryonated chicken eggs, cell culture and mice have been influenced by stalk length (Castrucci et al. 1993). Viruses with long stalks grew to higher titres in embryonated chicken eggs than those with shorter NA stalks. Furthermore, viruses with short stalks can maintain their virulence in humans and poultry (Landolt and Olsen 2007).
2.1.4.3 Polymerase complex (PA, PB1 and PB2)

The influenza A viral trimeric polymerase complex is often involved in viral replication and interaction with host factors (Subbarao et al. 1993; Naffakh et al. 2000). Temperature at the site of replication could make an impact on the function of the polymerase complex, thereby determining the host tropism of influenza virus (Baigent and McCauley 2003). Attenuation of virulence or replicative ability in specific hosts has been influenced by reassorted constellations of these three polymerase genes, sometimes also including NP (Webster et al. 1992). Amino acid substitutions in the polymerase proteins may impact host range by modifying interactions of the polymerase complex with host cell factors (Landolt and Olsen 2007).

The effects of the PB2 gene on species specificity have been demonstrated (Webster et al. 1992). Additional amino acid residues in PB2 could contribute to host range restriction (Penn 1989). Furthermore, the mutation at residue 627 of the PB2 gene has been hypothesised as a key determinant of influenza host range (Landolt and Olsen 2007). Regarding PB1, their effects on host range have also been identified as substitution of avian for human PB1 in a human virus attenuates its replication in MDCK cells and squirrel monkeys but not in chicken kidney cells (Webster et al. 1992).

2.1.4.4 Nucleoprotein (NP)

It has been proposed that the NP gene is a significant determinant of host range by restricting or attenuating virus replication, thereby controlling the successful
transmission of virus to a new host (Scholtissek et al. 1985; Tian et al. 1985; Snyder et al. 1987; Brown, 2001; Abe et al. 2004). The phosphorylation pattern of the NP protein could determine the extent to which a particular cell line supports virus growth (Kistner et al. 1985). Moreover, sequence analyses have demonstrated that the NP gene has evolved into five distinct, host-specific lineages: two equine lineages, one pig and human lineage, one aquatic bird lineage and one lineage in other avian species (Webster, 1992).

2.1.4.5 M Segment

Recently, it has been proposed that the M segment of the A(H1N1)pdm09 virus appears to be a critical factor for supporting transmissibility in humans (Torremorell et al. 2012). It was found that the reassortant H3N2 swine-origin influenza viruses detected in children from various states in the USA contained the M segment of the A(H1N1)pdm09 virus (CDC 2011b). Compared to precursor strains from the triple reassortant H3N2 SIV lineage, the M segment may promote efficient human-to-human transmission and also may enhance the rapid dissemination to other species (Torremorell et al. 2012).

2.1.4.6 Nonstructural proteins (NS)

The NS1 protein cooperates with a number of cellular factors associated with mRNA processing that may have an effect on species specificity (Landolt and Olsen 2007). In addition, the protein is involved in the initial host immune responses (Neumann and Kawaoka 2006).
2.2 History of swine influenza viruses

2.2.1 Classical H1N1 virus

Swine influenza (SI) was first detected in the USA during the devastating 1918 human influenza pandemic, which killed nearly 700,000 Americans and 40 million people worldwide (Reid and Taubenberger 2003). The first cases of SI were seen on farms in Western Illinois in August 1918 (Shope 1964). The coincidence and the similarity of signs and symptoms in humans and pigs were observed by Dr. Koen who believed that the two diseases were identical and called the new disease in pigs “flu” (Dorset et al. 1922). Signs of the disease in both pigs and humans were nasal discharge, coughing, fever, labored breathing and conjunctivitis. In addition, pneumonia can be induced after intratracheal administration of the virus (Winkler and Cheville 1986).

The swine influenza virus (SIV) was initially isolated by Shope (1931) in 1930. The prototype strain of the SIV was characterised as a type A subtype H1N1 virus that was the progenitor of the so-called “classical” H1N1 lineage of swine influenza A viruses. The genetic and antigenic characteristics of the virus were indistinguishable from the type A influenza virus responsible for human pandemics (Yoon et al. 2002). Genetic sequencing studies of the HA gene of the pandemic virus revealed that the virus most likely spread from humans to pigs. That was consistent with observations from veterinarians who described the disease in pigs after its occurrence in humans (Brown 2000).

After its first appearance, annual outbreaks of SI occurred during the winter months and were restricted to the north and the mid-west of the USA (Yoon et al. 2002).
Prior to 1975, there were only a few cases of SI in other countries, apart from the USA (Easterday and Van Reeth 1999). In Europe, the SIV was isolated in the UK (Blakemore and Gledhill 1941) and Czechoslovakia (Harnach et al. 1950), while antibodies to H1N1 influenza viruses were found in pigs in Germany (Kaplan and Payne 1959). There was no report of SIV cases in Europe for almost 20 years, until 1976 when clinical SI reemerged on farms in northern Italy (Brown 2000). The virus causing these outbreaks was closely related to classic H1N1 virus and it was hypothesised that the virus was introduced into Italy via a shipment of pigs from the USA (Nardelli et al. 1978). The disease was confined to northern Italy until 1979. Subsequently classical SIV was reported in Belgium (Vandeputte et al. 1980) and France (Gourreau et al. 1980). The disease spread quickly to other European countries including the Netherlands (Masurel et al. 1983), Germany (Witte et al. 1981), Denmark (Sorensen et al. 1981), Sweden (Martinsson et al. 1983) and the UK (Roberts et al. 1987).

Prior to 1998, all of the RNA segments of the “classical” H1N1 virus were of swine origin as it was initially isolated from pig populations in the USA in the early 1930s (Ma et al. 2006). However, the predominant H1N1 SIV currently isolated in the USA is a reassortant H1N1 virus, containing RNA segments (PA and PB2) of avian origin. Moreover, a reassortant H1N1 virus carrying internal proteins from triple reassortant H3N2 viruses became prominent among pigs in North America (Janke 2004). Other variants in pigs have also been demonstrated. For example, a reassortant H1N1 virus in Canada consisted of classical SIV genes and a human PB1 polymerase gene (Karasin et al. 2006). In China, a wholly human lineage H1N1 virus was found in pigs in 2007 (Yu et al. 2007).
2.2.2 Human-like H3N2 virus

In 1970, Hong Kong H3N2 virus from humans was first isolated from pigs in Taiwan. In 1984, the first case of swine H3N2 virus was reported in continental Europe (Haesebrouck et al. 1985) and a few years later it was detected in Great Britain (Wibberley et al. 1988). Following these initial detections, the “human-like” H3N2 virus continued to circulate in European pig populations long after its disappearance from human populations. Clinical outbreaks caused by the “human-like” H3N2 virus were usually seen with a high seroprevalence in pigs in Europe (Brown 2000). In contrast, a low prevalence of the H3N2 subtype was reported in pigs in North America, suggesting that the virus was not established in the American pig population and infection occurred rarely following introduction from infected humans (Easterday 1980).

In 1998, novel H3N2 SIVs (double-reassortant H3N2 viruses) were isolated from pigs with respiratory signs in North Carolina. The HA, NA and PB1 segments of the viruses were of human origin and other gene segments from classical H1N1 SIV. The situation of SIVs in the USA then changed after the subsequent isolation of H3N2 viruses from other states. These were triple-reassortant H3N2 viruses with a combination of human, swine and avian influenza virus genes (Zhou et al. 1999). To illustrate, the HA, NA and PB1 segments of the viruses were from human H3N2 influenza virus; the M, NS1 and NP segments were of classical swine H1N1 origin; and the PA and PB2 segments were from avian viruses (Webby et al. 2000). Subsequently the triple-reassortant H3N2 viruses have become widespread in the pig population in North America.
2.2.3 Avian-like H1N1 virus

Since 1979 “avian-like” H1N1 virus has been the dominant H1N1 virus in European pigs. It is genetically and antigenically different from classical H1N1 SIV, but related closely to H1N1 viruses isolated from ducks (Pensaert et al. 1981). All gene segments of the prototype virus were of avian origin (Schultz et al. 1991). After 2 years of the introduction of the “avian-like” virus into pigs in Great Britain, classical H1N1 virus apparently disappeared as a clinical entity (Brown 2000). It appeared that the “avian-like” virus has a selective advantage over classical SIV. In Europe, the virus has replaced the previously circulating classical H1N1 SIV and been co-circulating with H3N2 viruses (Campitelli et al. 1997). In South East Asia, the “avian-like” H1N1 virus was detected in pigs and since 1993 the virus has been co-circulating with classical H1N1 virus (Guan et al. 1996).

2.2.4 H1N2 virus

In the late 1980s, the first H1N2 influenza virus was isolated from pigs in France. The virus descended from classical H1N1 and “human-like” H3N2 SIVs (Gourreau et al. 1994) with the HA gene being derived from the classical H1N1 virus and the NA gene from the swine-adapted human virus. Since 1994, H1N2 subtype has become endemic in pigs in Great Britain and subsequently spread to pigs in the rest of Europe (Brown et al. 1995a; Van Reeth et al. 2000). It has been reported that H1N2 reassortants have been isolated from pigs in France (Gourreau et al. 1994), Belgium (Van Reeth et al. 2000), the UK (Brown et al. 1998) and Japan (Ito et al. 1998). In particular, the H1N2 viruses in Japan and the United Kingdom caused large-scale outbreaks of disease in pig populations in the region (Brown et al. 1998).
In North America, the first isolation of H1N2 subtype from pigs was reported in Indiana in 1999 (Karasin et al. 2000a). Phylogenetic analyses demonstrated that the H1N2 virus inherited HA gene from a virus of the classical H1 swine lineage and the remaining genes from the triple-reassortant H3N2 viruses recovered from pigs in the USA in 1998-1999. Following this first isolation, H1N2 viruses have been isolated from pigs in six states of the USA. The viruses have become established and have been circulating together with H1N1 and H3N2 viruses in pig populations of the USA.

Other variants of H1N2 subtype have been detected in pigs from several countries. In Canada, the H1N2 viruses containing NA and HA genes from two different human influenza viruses, polymerase gene from human H1N2 viruses and other internal genes from classical H1N1 influenza virus, have been isolated (Karasin et al. 2006). The H1N2 viruses in European pigs were derived by reassortment between human H1N1 viruses and “human-like” European H3N2 viruses (Heinen 2003). In China, the H1N2 strain from North America and the H1N2 reassortants between classical H1N1 viruses and North American H3N2 human influenza viruses have been reported (Xu et al. 2009).

2.2.5 H3N1 virus

Recently, novel swine influenza H3N1 viruses were isolated in Taiwan, the USA and Korea (Lekcharoensuk et al. 2006; Ma et al. 2006; Shin et al. 2006). Phylogenetic analyses demonstrated that these H3N1 viruses were derived by reassortment between H1N1 and H3N2 SIVs. Furthermore, new reassortant H3N1 viruses were isolated from coughing pigs in the USA. In 2006, the reassortant H3N1 subtype
derived from the circulating H3N2 and H1N1 SIVs was identified in Italy (Moreno et al. 2009).

2.2.6 A(H1N1)pdm09 virus

In late March 2009, 2 children living in adjacent counties in southern California had an onset of acute febrile respiratory illness. Clinical specimens from both children were tested at local laboratories and influenza A viruses isolated. However, as no specific subtype could be identified the specimens were tested further at reference laboratories. By mid-April, atypical cases and clusters of severe pneumonia were appearing principally in previously healthy young adults in different areas in Mexico. On 15 and 17 April 2009, the United States Center for Disease Control and Prevention (CDC) declared that these 2 children were infected with a novel virus that contained genetic material suggestive of swine origin. However, the virus had not been detected in pigs or humans before. The children had neither been exposed to pigs nor had contact with each other (WHO 2009a).

The A(H1N1)pdm09 virus had spread so quickly and extensively among humans around the world, until June 11, the WHO declared a phase 6 pandemic - the first influenza pandemic in the past 41 years. The A(H1N1)pdm09 virus spread within 6 weeks since the first cases were detected in California in 2009 (WHO 2009b). Phylogenetic analyses of the A(H1N1)pdm09 virus isolated are illustrated in Figure 2.3. The virus is a genetic reassortment of four different influenza virus strains including: 1) human influenza gene segment; 2) avian gene segments from North America; 3) swine influenza gene segments from North America; and 4) Eurasian avian-like swine gene segments (Girard et al. 2010). The triple reassortant containing
genes of the first three virus strains were initially seen and had been circulating in pigs at least ten years prior to the onset of human cases. It can cause occasional and mild disease in humans in close contact with pigs. This combination of gene segments between the first triple reassortment and the Eurasian pig influenza virus had never been previously reported either in pigs or in humans (Ferrari et al. 2009).

**Figure 2.3.** History of reassortment events in the evolution of the A(H1N1)pdm09 virus (Trifonov et al. 2009).
After the detection of the A(H1N1)pdm09 virus in humans, the virus then spread quickly around the world, causing disease in humans and other animal species (Torremorell et al. 2012). In May 2009, the A(H1N1)pdm09 virus was firstly isolated from a pig herd in Alberta, Canada. It was most likely that pigs were exposed to the virus from a Canadian who had recently returned from Mexico and had been exhibiting flu-like symptoms (Pasma and Joseph 2010). Soon after the first case in pigs in Canada, the A(H1N1)pdm09 virus was identified in pig populations worldwide (Moreno et al. 2010; Pasma and Joseph 2010; Pereda et al. 2010; Song et al. 2010; Welsh et al. 2010). Although human-to-pig transmission was suspected in most cases, definitive evidence was lacking (Torremorell et al. 2012). Previous studies demonstrated that pigs were highly susceptible to infections with the A(H1N1)pdm09 virus, suggesting that the virus could become readily established in pig populations following primary contact with infected humans (Lange et al. 2009; Brookes et al. 2010).

### 2.2.7 Swine influenza viruses in Thailand

The subtypes of H1N1, H3N2 and H1N2 SIVs have been circulating in the Thai pig population for decades (Takemae et al. 2008). Historically, the first subtype was H3N2 virus isolated from Thai pigs in 1978 (Nerome et al. 1981). The swine H3N2 virus was shown to be closely related to contemporary human viruses, both antigenically and genetically, suggesting the frequent transfer of human influenza viruses to a pig host (Nerome et al. 1981). Later in 1988, the first H1N1 subtype was detected from pigs in Chonburi province during the early febrile stage of an influenza-like illness (Kupradinun et al. 1991). The isolation of swine H1N1 viruses in Thailand provided evidence for the introduction of A/NJ/8/76-like variants
through pigs imported from the USA. Later in 2005, the emergence of a new H1N2 subtype was reported (Damrongwatanapokin et al. 2006).

The co-circulation of three subtypes of SIVs (H1N1, H1N2 and H3N2) in the pig population of Thailand could potentially facilitate the opportunity of evolutional events of SIVs. Recent phylogenetic analysis of Thai SIVs revealed the existence of nine distinct genotypes that arose from multiple introductions of classical swine, avian-like swine and human viruses (Takemae et al. 2008). On 14 December 2009, the first case of the A(H1N1)pdm09 virus was confirmed in Thai pigs in Saraburi province (OIE 2009).

2.3 Geographic distribution of virus infections

Swine influenza is endemic in most densely swine-populated regions (Van Reeth 2007). The disease is found in pigs across the world such as the mid-western USA (and occasionally in other states), Mexico, Canada, South America, Europe (including the UK, Sweden and Italy), Kenya, China, Japan, Taiwan and other parts of eastern Asia (Merck & Co. 2006). However, the situations for SIV infections vary between continents (CFSPH 2009). Outbreaks of SI most commonly occur in winter or fall in North America. In warmer areas, pig infections with SIVs may be present throughout the year (Merck&Co. 2006).

2.3.1 Europe

The classical H1N1 virus was endemic in pigs in Europe, with a seroprevalence of 20% to 25% (Brown et al. 1995b). However, after the emergence of "avian-like" H1N1 virus, the main H1N1 subtype circulating in European pigs became “avian-like” H1N1 virus. Serologic examination of finishing pigs presented the prevalence
of the H1N1 and H3N2 strains at 92 and 57% in Belgium (1996); 73 and 62% in Spain (1992); 55 and 51% in Germany (1993); and 60 and 30% or 54 and 13% in the Netherlands (1990), respectively (Heinen 2003). Serosurveillance results in Great Britain demonstrated that more than half of adult pigs in the national population had been infected with one or more influenza A viruses during their lifetime, particularly 14% of pigs had been infected with influenza viruses of both human and swine origins (Brown et al. 1995b). In Belgium, H1N1 seropositivity in pigs in 1998 was at 100% of farms examined, while antibodies against H3N2 and H1N2 were found in 97.3 and 85% of studied farms, respectively (Van Reeth et al. 2000).

A Spanish seroprevalence study reported that 93 out of the 98 participating farms had antibodies to influenza viruses. Specifically, antibodies against subtypes H1N1, H1N2 and H3N2 were detected in 92.9, 64.3 and 92.9% of farms, respectively. In addition, antibodies to more than one subtype were found in 87.8% of the studied farms (Simon-Grife et al. 2011). A British seroprevalence study concluded that 52% of the participating farms had antibodies to at least one subtype (Mastin et al. 2011). Van Reeth et al. (2008) conducted a seroprevalence study in seven European countries including Belgium, the Czech Republic, Germany, Italy, Ireland, Poland and Spain. All seven countries contained pigs that tested positive for influenza antibodies; however, the Czech Republic, Ireland and Poland had a lower seroprevalence compared to the other participating countries. Overall, the H1N1 subtype was the most widespread followed by H1N2 and H3N2 subtypes.
2.3.2 North America

Surveillance data in North America in the 1990s demonstrated that classical H1N1 influenza viruses were the primary cause of influenza infections among pigs in the USA. In the north-central USA, previous studies demonstrated widespread exposure to H1 influenza viruses of pigs, with H1 seropositivity rates of 47% in 1976-1977 (Hinshaw et al. 1978), 51% in 1988-1989 (Chambers et al. 1991) and 28% in 1997-1998 (Olsen et al. 2000), respectively. Approximately 25-33% of 6-7 month-old finishing pigs and 45% of breeding pigs in the USA had antibodies to the classical swine H1N1 virus, while seropositivity to H3 subtype viruses was very limited (at 1.4 and 1.1%, respectively) (CFSPH 2009).

A seroprevalence study conducted in the USA reported that 22.8% of pigs had antibodies to H1N1 or H3N2 (Choi et al. 2002). Sixty-six per cent of the total number of positive samples were accounted for by H1N1 infections, whereas the remaining positive samples were caused by H3N2 subtype. In Canada, prevalence studies in Ontario concluded that the sows and finishing pigs tested were positive to H1N1 virus at 83.1 and 40.3%, respectively (Poljak et al. 2008a). In 2003, sows were tested positive for antibodies to the Colorado and Texas strains of H3N2 at 9.2 and 7.9%, respectively (Poljak et al. 2008a). In the following year, seroprevalence against H1N1 and H3N2 subtypes in finishing pigs accounted for 13.4 and 2.7%, respectively. Later in 2005, the prevalence of H1N1 and H3N2 increased to 14.9 and 25.9%, respectively (Poljak et al. 2008b).
2.3.3 Asia

In Asia, the classical H1N1 virus, “avian-like” H1N1 virus and “human-like” H3N2 virus have been identified (Reperant et al. 2009). The prevailing influenza virus infections in Asian pigs are classical H1N1 viruses (Guan et al. 1996). In the pig population in China, three subtypes of H1N1, H1N2 and H3N2 have been co-circulating (Bi et al. 2010). Serological studies have been carried out in China using the haemagglutination inhibition (HI) assay with H1, H3, H5 and H9 subtype influenza viruses as antigens. The percentage of pigs positive to H1 and H3 subtype were 10.1 and 41.1%, respectively. In addition, some samples were positive to H9 (1.9 to 6.8%). Importantly, H5 subtype was detected from two separate farms at a prevalence of 3.9 and 9.5%, respectively (Li et al. 2004). A pooled data analysis of antibodies against influenza viruses over a 10-year period showed the average prevalence of H1, H3, H5, H7 and H9 in Chinese pigs at 31.1%, 28.6%, 1.3%, 0% and 2.4%, respectively (Liu et al. 2011).

In Korea, H1N1, H1N2 and H3N2 subtypes are currently co-circulating in pigs. The seroprevalence of antibodies against the swine H1 viruses, H3 viruses, and both H1 and H3 viruses were 51.2, 43.7 and 25.3% respectively in a study reported by Jung et al. (2007). A Korean study conducted by Pascua et al. (2008) demonstrated that 2,959 out of 6,418 growing-finishing pig sera (46.1%) had antibodies against influenza A viruses. It was estimated that 41.5% of the samples had antibodies for H1, whereas only 3.7% had antibodies for H3; and 0.9% of the samples tested positive for both subtypes. A study in Malaysia presented that the seroprevalences of H1N1 and H3N2 in pigs were 12.2 and 12.1%, respectively (Suriya et al. 2008).
Seropositivity for either of the virus subtypes was detected at 41.4% of sampled farms. A combination of both subtypes was detected in 4% of all pigs and in 22% of sampled farms.

In Thailand, SIV infections have been shown to be widespread. During 2004, the percentages of seropositive pigs against H1N1 and H3N2 influenza viruses by HI test were 14.1 and 22.1%, respectively (Parchariyanon 2006). A serological study was conducted in fattening and breeding pig farms in Thailand from 2004 to 2005 (Damrongwatanapokin et al. 2006). It was found that 37% of fattening farms (27/70) and 81.4% of breeding farms (74/88) were seropositive to both H1N1 and H3N2 subtypes (Damrongwatanapokin et al. 2006). A study by Nakharuthai et al. (2008) demonstrated the occurrence of SIV infection with porcine respiratory disease complex (PRDC) in piglets. It presented that 3 of 30 farms (10%) were positive to SIVs. Furthermore, SIVs were detected in 3 of 106 samples (2.8%) (Nakharuthai et al. 2008).

2.4 Epidemiology of swine influenza viruses

2.4.1 Reservoirs of influenza A viruses

Influenza A viruses can infect multiple species including humans, pigs, horses, sea mammals and birds (Webster et al. 1992). In particular, wild aquatic birds have been proposed to be the primordial reservoir of all influenza viruses from avian and mammalian species (Webster et al. 1992). Given their migratory behaviour and the ability of influenza viruses to persist in cold lake water, waterfowl are significant reservoirs for influenza viruses in nature (Heinen 2003). All 16 HA and 9 NA
subtypes have been found in wild waterfowl and seabirds (Vincent et al. 2008). Most influenza viruses are nonpathogenic for wild birds and tend to persist in avian populations (Yoon et al. 2002). Importantly, feral ducks have a potential to disseminate influenza viruses over large areas and across great distances (Yoon et al. 2002).

2.4.2 Transmission routes

The primary route of virus transmission between pigs is through direct contact via the nasopharyngeal route, particularly through nose-to-nose contact or contact with secretions containing SIVs, e.g. nasal discharges of infected pigs during the acute febrile stages, providing a massive source of infectious materials to infect susceptible animals (Easterday and Van Reeth 1999).

2.4.3 Interspecies transmission

There is substantial evidence demonstrating that interspecies transmission of influenza viruses can occur (Brown, 2000).

2.4.3.1 Transmission from humans to pigs

In the 1918 pandemic, H1N1 human influenza virus was transmitted among people and pigs. Genetic sequencing of the human virus along with the chronology of the epidemics in human populations and pigs suggested that the viruses were transmitted from humans to pigs (Brown 2000). Shope (1938) presented serological evidence that human-to-pig transmission could occur. However, the first case of pigs infected with human viruses was confirmed in Taiwanese pigs that were infected with Hong Kong H3N2 influenza virus (Kundin, 1970). There is no apparent evidence of pigs being infected with this subtype prior to the pandemic in humans in 1968. Indeed,
the appearance of a H3N2 subtype variant strain in the pig population appeared to coincide with the epidemic strain infecting the human population at that time (Nerome et al. 1981; Brown et al. 1995b).

Following genetic reassortment, potential transmission of human influenza virus genes to pigs could occur. For example, genetic analysis of two strains of H1N1 viruses isolated from pigs in Japan suggested that the HA and NA genes were most closely related to those of human H1N1 viruses circulating in the human population at that time (Katsuda et al. 1995). In Great Britain, reassortant H1N2 influenza viruses from pigs have been identified. The HA is related most closely to that of a human H1 virus from the early 1980’s (Brown et al. 1998). In Thailand, it was found that H3N2 isolates acquired the HA gene by the introduction of human H3N2 virus into the Thai pig populations (Takemae et al. 2008; Lekcharoensuk et al. 2010). Recently, the A(H1N1)pdm09 virus was first detected in Canadian pigs after widespread spread in human populations across the world. Human-to-pig transmission was suspected in most of the initial pig farm cases (Torremorell et al. 2012).

2.4.3.2 Transmission from pigs to humans

In 1974, SIV was first isolated from a human (Smith et al. 1976). Later in 1976, a swine-origin influenza virus of H1N1 subtype (A/New Jersey/76) was isolated from soldiers during the so-called “New Jersey” incident at Fort Dix in the USA (Gaydos et al. 1977, 2006). Following a respiratory disease outbreak, one soldier died and 12 additional soldiers were hospitalized due to influenza. Pigs were contended to be the
source of infection as the causative viruses were identical to viruses isolated from pigs in the USA (Brown 2000). However, the definitive source of the virus at Fort Dix is unknown and no exposure to pigs was ever documented (Gaydos et al. 2006).

Evidence of the zoonotic nature of SIVs emerged in November 1976 when H1N1 SIVs were isolated from pigs and their caretaker from a farm in southern Wisconsin (Hinshaw et al. 1978). Pigs had been sick with classic clinical signs of SI for 2 or 3 days, while one of the caretakers also became ill with moderate to severe influenza symptoms (Easterday and Van reeth 1999). The characterisation of the viruses from the human and the pigs were identical (Hinshaw et al. 1978). Most SIV infections in humans are not clinically different from human influenza virus infections (Van Reeth 2007), but fatal cases can occur. Occasionally SIV have been isolated from the respiratory secretions or lungs of people from Europe, Asia and the USA (see Table 2.1 for details) (Van Reeth 2007).
Table 2.1. Swine influenza viruses isolated from humans worldwide (Van Reeth 2007).

<table>
<thead>
<tr>
<th>Continent</th>
<th>Year</th>
<th>Country/ State</th>
<th>Virus</th>
<th>Patient</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europe</td>
<td>1986</td>
<td>The Netherlands</td>
<td>H1N1</td>
<td>3 young men of whom 2 in close contact with pigs</td>
<td>Mild respiratory disease (2 patients) and severe pneumonia (1 patient) Severe pneumonia</td>
</tr>
<tr>
<td></td>
<td>1993</td>
<td>The Netherlands</td>
<td>H1N1</td>
<td>Girl living on farm (5 years)</td>
<td>Severe pneumonia</td>
</tr>
<tr>
<td></td>
<td>1993</td>
<td>The Netherlands</td>
<td>H3N2</td>
<td>2 children (1 and 2 years)</td>
<td>Mild respiratory disease</td>
</tr>
<tr>
<td></td>
<td>2002</td>
<td>Switzerland</td>
<td>H1N1</td>
<td>Swine farmer (50 years)</td>
<td>Influenza symptoms</td>
</tr>
<tr>
<td>Asia</td>
<td>2002</td>
<td>Hong Kong</td>
<td>H3N2</td>
<td>Child (10 months)</td>
<td>Mild respiratory disease</td>
</tr>
<tr>
<td>North America</td>
<td>1975</td>
<td>Texas</td>
<td>H1N1</td>
<td>Boy (13 years) with Hodgkin’s disease</td>
<td>Fatal pneumonia</td>
</tr>
<tr>
<td></td>
<td>1976</td>
<td>New Jersey</td>
<td>H1N1</td>
<td>Approx. 500 people infected</td>
<td>1 fatal case</td>
</tr>
<tr>
<td></td>
<td>1979</td>
<td>Texas</td>
<td>H1N1</td>
<td>Student (20 years) after working at a swine livestock show</td>
<td>Influenza symptoms</td>
</tr>
<tr>
<td></td>
<td>1980</td>
<td>Texas</td>
<td>H1N1</td>
<td>Boy (6 years) after visiting a regional livestock show</td>
<td>Influenza symptoms</td>
</tr>
<tr>
<td></td>
<td>1982</td>
<td>Nevada</td>
<td>H1N1</td>
<td>Girl (5 years) with leukemia</td>
<td>Fatal pneumonia</td>
</tr>
<tr>
<td></td>
<td>1988</td>
<td>Wisconsin</td>
<td>H1N1</td>
<td>Woman (32 years, 3rd trimester of pregnancy) after visiting a pig fair</td>
<td>Fatal pneumonia</td>
</tr>
<tr>
<td></td>
<td>1991</td>
<td>Maryland</td>
<td>H1N1</td>
<td>Laboratory animal caretaker (27 years) in contact with sick pigs</td>
<td>Fatal pneumonia</td>
</tr>
<tr>
<td></td>
<td>1995</td>
<td>Minnesota</td>
<td>H1N1</td>
<td>Woman (37 years) working in a swine farm</td>
<td>Fatal pneumonia</td>
</tr>
<tr>
<td></td>
<td>1997</td>
<td>Wisconsin</td>
<td>H1N1</td>
<td>2 animal caretakers (31 and 39 years) exposed to experimentally infected pigs</td>
<td>Influenza symptoms</td>
</tr>
</tbody>
</table>

*a All H1N1 viruses isolated from humans in Europe were avian-like; H1N1 viruses isolated from humans in the USA were “classical”.*

31
In addition, serological evidence of human infections with swine-origin influenza viruses has been demonstrated in several studies (Olsen et al. 2002; Myers et al. 2006; Terebuh et al. 2010; Gerloff et al. 2011). In a study conducted in Wisconsin, seropositivity to H1N1 SIV was significantly associated with being a farm owner or a farm family member, living on a farm or entering the swine barn ≥ 4 days/week (Olsen et al. 2002). Humans with occupational exposure to swine such as farmers, veterinarians and meat processing workers were shown to be at higher odds of exposure to swine H1N1 and H1N2 viruses compared with controls (Myers et al. 2006). Furthermore, swine workers and their non-exposed spouses were found to be at increased risk of SIV infection (Gray et al. 2007).

A prospective cohort study presented serologic evidence for infection with human and swine-origin influenza viruses in swine workers in the USA (Terebuh et al. 2010). A low rate of symptomatic infection and virus isolation among participant swine workers was also identified in that study. In Europe, swine workers were shown to have more frequent and higher titres to swine influenza viruses compared to controls (Gerloff et al. 2011). In Thailand, the serological evidence of pig-to-human influenza virus transmission was observed among swine farm workers (Kitikoon et al. 2011b). Recently, reassortant H3N2 swine-origin influenza viruses with the matrix gene segment of the A(H1N1)pdm09 virus were isolated from children in various states in the USA (CDC 2011b). Most humans infected with swine-origin influenza viruses have reported previous direct or indirect exposure to pigs and the course of disease is similar to that of typical influenza virus infections. However, some of these recent cases also suggest limited human-to-human transmission (CDC 2011a).
2.4.3.3 Transmission from birds to pigs

Infections of pigs with avian influenza viruses have been reported. Based on phylogenetic analyses, most of the infections were thought to originate from wild ducks (Torremorell et al. 2012). In Europe, avian H1N1 viruses were transmitted to pigs in 1979 and became established as a stable lineage that is still circulating in pig populations (Pensaert et al. 1981; Kyriakis et al. 2011). Thereafter, numerous laboratories have isolated wholly avian influenza viruses (AIVs) from pigs (Ma et al. 2009). Kida et al. (1994) demonstrated that pigs could be infected with the H1-H13 subtypes of AIVs and may also be susceptible to H14-H16 subtypes. The results from these experiments further showed that avian viruses do not replicate in pigs, but they can contribute genes in the generation of reassortants when pigs are co-infected with SIVs (Brown 2000). In 1993, an avian H1N1 virus was isolated from Chinese pigs (Guan et al, 1996). In Canada, H4N6, H3N3 and H1N1 AIVs were detected from pigs in 1999, 2001 and 2002, respectively (Karasin et al. 2000b; Karasin et al. 2004).

Ninomiya et al. (2002) provided serological evidence of infection with H4, H5 or H9 AIVs in Asian pigs. Recently, an H9N2 AIV from poultry was isolated from pigs in several provinces in China (Xu et al. 2004; Cong et al. 2007; Yu et al. 2007). Importantly, H5N1 highly pathogenic avian influenza (HPAI) virus was identified in pigs in Asian countries (Zhu et al. 2008). There has also been serologic and virologic evidence of pigs infected with H5N1 in Vietnam and Indonesia. It is conceivable that pigs can serve as direct and intermediate hosts for many subtypes of AIVs (Ma et al. 2009). However, recent studies have demonstrated that domestic pigs show only low susceptibility to H5N1 HPAI virus (Choi et al. 2005; Lipatov et al. 2008). In
addition, experimental data has indicated that pig-to-pig transmission of the virus has not occurred (Choi et al. 2005; Lipatov et al. 2008; Nidom et al. 2010).

2.4.3.4 Transmission from pigs to birds

Among domestic avian species, turkeys represent the species that is most susceptible to SIV infections (Torremorell et al. 2012). The potential introduction of classical swine H1N1 viruses from infected pigs to turkeys was firstly documented in North America (Mohan et al. 1981). To illustrate, after introduction of boars into a herd, two outbreaks of respiratory disease were observed and subsequently there was a drop in egg production in the turkey flocks associated with the pig herd. Diagnostic test results concluded that both species had been infected by the same H1 influenza virus (Mohan et al. 1981). Since then there have been numerous reports showing that influenza viruses can be isolated from turkey flocks and were characterised to be swine-origin influenza viruses (Andral et al. 1985; Ficken et al. 1989; Suarez et al. 2002; Choi et al. 2004; Tang et al. 2005).

2.4.4 Potential role of pigs as intermediate hosts in the creation of pandemic viruses

Pigs have been suggested as potential “mixing vessels” of influenza viruses from different sources for a long time. Pigs are likely to be the permissive host of influenza viruses as they have a broader host range in the compatibility of the NP gene in reassortant viruses than both humans and birds (Brown 2001). In particular, the pig trachea contains epithelial cells with both receptors that avian and human influenza viruses preferentially bind to, facilitating reassortment events of influenza viruses from different origins (Ito et al. 1998). In addition, conversion of the receptor
specificity of influenza viruses could occur in pigs. To illustrate, continued passage of avian viruses within pigs could potentially lead to alterations of the receptor binding specificities by modifying a former α-2,3 linkage to a α-2,6 linkage, which is the native linkage in humans (Brown 2001). The avian viruses circulating within pig populations could thus become more ‘human-like’ viruses in pigs. This is crucial for generating pandemic influenza viruses in humans.

Supportive evidence for the “mixing vessels” theory has been demonstrated in Europe by Castrucci et al. (1993) who detected reassortment of human and avian viruses in pigs from Italy (Brown 2000). Moreover, the H1N2 virus, derived from a multiple reassortant event, spread widely within pig populations in Great Britain (Brown 2000). A H1N7 subtype derived from human and equine viruses has also been isolated from pigs in Great Britain and H2N3 reassortant viruses have been shown to contain genes derived from avian and swine influenza viruses (Ma et al. 2007). Recently double (avian/human; human/swine) and triple (human/avian/swine) reassortant influenza A viruses were isolated from pigs in the USA and China (Ma et al. 2009).

2.4.5 Risk factors for transmission of influenza A viruses

Both sick and subclinically infected pigs are likely to play an important role in the transmission of influenza viruses within and between herds (Torremorell et al. 2012). Generally, influenza appears when infected pigs are introduced into a herd, either through the movement or mixing of infected pigs with susceptible animals. Once a herd is infected, the virus persists through infection of young susceptible pigs or
through infection of susceptible new stock. Affected pigs may display clinical signs and the disease can spread rapidly resulting in it becoming endemic (Brown 2000).

At the herd level, various risk factors for influenza virus infections have been identified. The most common risk factors across different pig production regions include high pig and/or farm density, large herd size, high replacement rates and the importation or purchase of pigs (Ewald et al. 1994; Maes et al. 2000; Poljak et al. 2008a; Suriya et al. 2008; Mastin et al. 2011; Simon-Grife et al. 2011). Tielen et al. (1978) reported that the number of pigs per barn influenced the likely occurrence of infection. This was confirmed by Elbers et al. (1991) who observed a negative effect on respiratory health when there were >100 pigs in one compartment. Flesja et al. (1982) also reported that when pens contained more than 12 pigs there was a negative effect on respiratory health. The study by Maes et al. (2000) also demonstrated that the number of pigs per pen was positively associated with swine influenza H3N2 seropositivity. A large number of pigs per pen allows for more opportunities for direct nose-to-nose contact or for aerosol spread of influenza viruses, as well as physiological stress that can alter the immune system and predispose pigs to virus infection (Maes et al. 2000).

Increased replacement rates were identified as one of the risk factors for SI (Simon-Grife et al. 2011). To illustrate, replacement rate reflects the number of replacement gilts introduced onto farms. The replacement gilts could act as a source of susceptible animals, as well as a source of subclinically infected animals. Rosendal and Mitchell (1983) considered the number of pigs purchased at one time as an
important factor since the likelihood of buying one infected animal increases with the number of animals purchased. In Malaysia, the seropositivity to H1N1 and H3N2 viruses in pigs was significantly associated with importation or purchase of pigs (Suriya et al. 2008). In particular, larger scale farms in Malaysia mostly imported pigs originating from European countries where both H1N1 and H3N2 SIVs are common.

Other farm management practices have also been associated with herd infections. It was suggested that exclusively fattening farms had a higher risk of acquiring respiratory diseases than exclusively breeding or mixed breeding–fattening farms (Stark 2000). In fattening farms growing pigs and larger numbers of finishing pigs are frequently purchased from different sources or from a market where the risk of infection is highest. In contrast, breeding farms are less likely to buy large numbers of animals from several sources (Stark 2000). The study by Trevennec et al. (2012) also identified farms specialized in fattening as a risk factor of SIV infections in pigs in Vietnam. In contrast, Simon-Grife et al. (2011) showed that solid pen separations seemed to be associated with lower prevalences against influenza viruses in fattening pigs.

Recently, a British study concluded that the number of pigs per water space and indoor housing was positively associated with an increased probability of virus circulation (Mastin et al. 2011). A large number of pigs per water source may contribute to increase in both direct and indirect contact between pigs, as well as induce stress amongst pigs. Indoor pen-based systems may also contribute to higher
stocking densities and lower levels of ventilation, enhancing transmission of respiratory pathogens between pigs (Stark 2000). Suriya et al. (2008) found that the presence of pets, such as cats, on the farms enhances the risk of H1N1 and H3N2 infections in pigs, suggesting that mammalian pets play an important role in the spread and transmission of influenza virus.

It has previously been reported that farms located closely to one another had an increased possibility for windborne, personnel, fomites and disease transmission from one farm to another (Suriya et al. 2008). In particular, frequent contact between farms by vehicles could be associated with the substantial spread of influenza viruses. A higher number of pigs within the vicinity could facilitate airborne transmission and contacts between herds (Maes et al. 2000). Moreover, a number of studies confirmed that an increase in herd size and a decrease in the distance between neighbouring farms contributed to higher risks for virus infection (Stark et al. 1992; Thomsen et al. 1992). High pig density (≥ 200 pigs per km²) and large herd size (>300 finishing pigs) have been identified as factors related to the occurrence of influenza A virus infections in fattening pigs (Ewald et al. 1994). Furthermore, uncontrolled access to the farm was associated with higher seropositivity against H1N1 and seroprevalence against more than one subtype of influenza virus (Simon-Grife et al. 2011).

### 2.5 Pathogenesis

Infections with SIVs are largely restricted to the respiratory tract (Easterday and Van Reeth 1999). The viruses can replicate in epithelial cells of the whole respiratory tract including nasal mucosa, tonsils, trachea, tracheobronchial lymph nodes and
lungs (Easterday and Van Reeth 1999). In particular, the lungs appear to be the major target organ. There are no differences in the site of replication in the lungs with different SIV strains. A massive infection occurs in epithelial cells of the bronchi, bronchioles and alveoli (Easterday and Van Reeth 1999). The severity of illness appears to be determined by the amount of virus that reaches the deeper airways and the consequent production of infectious virus particles in the lungs (Easterday and Van Reeth 1999).

Following intratracheal inoculation of pigs, virus titres in the lungs may be greater than $10^8$ EID$_{50}$/gram of tissue (Haesebrouck and Pensaert 1986). Under experimental conditions, intranasal inoculation of high virus quantities ($10^7$-$10^{7.5}$ EID$_{50}$) in fattening pigs caused subclinical infections, whilst intratracheal inoculation at the same dose resulted in typical clinical signs within 24 hours of inoculation (Maes et al. 1984). Thus, typical disease and lung pathology only occur when pigs are experimentally inoculated with high virus doses directly via the intratracheal route.

In intratracheal infection studies, massive virus titres were found in the lungs and high levels of several cytokines or “signal molecules” were in lung lavage fluids (Van Reeth et al. 1998). The cytokines were interferon-alpha (IFN-α), tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and IL-6 that notably induced lung inflammation, functional lung disturbances, fever, malaise and loss of appetite. These cytokines can strongly enhance each other’s effects. After intratracheal inoculation with SIV, clinical signs were observed in pigs within 24 hours and were related to the peak virus replication and cytokine levels. In contrast, inoculation by the less invasive intranasal or aerosol routes caused lower virus titres in the lungs,
with the infections remaining clinically mild or subclinical and failing to stimulate the massive production of cytokines in the lung (Van Reeth 2007). After challenging vaccinated pigs with SIV, cytokine production has been shown to be greatly decreased or absent. This was related to the reduction in virus replication and hence disease prevention (Van Reeth et al. 2002). Thus, a number of factors, such as partial active immunity, passive immunity or sanitary measures, are likely to prevent disease through reducing the extent of virus replication.

Occasionally, SIVs have been isolated from extra-respiratory tissues. In an experimental study, 1 of 4 influenza viruses was isolated from the faeces of a single pig; however, virus replication in intestinal cells has never been demonstrated (Kawaoka et al. 1987). Isolation of the viruses from the serum of experimentally infected pigs has been reported 1 and 3 days post-challenge, although only in very low amounts (Brown et al. 1993). After day 7, SIVs could not be isolated from the lungs or other respiratory tract tissues of these challenged pigs (Brown et al. 1993). With the highly sensitive enzyme-linked immunosorbent assay (ELISA), influenza-specific antibodies could be detected in the serum on day 3 and from nasal swabs on day 4 post-challenge (Lee et al. 1995).

Virus has been detected in nasal swabs up to 6 to 7 days post-infection (Van Reeth 2007). In lung tissue the virus can undergo rapid replication and the highly specific tropism for bronchiolar epithelium by the virus has been demonstrated in studies using immunofluorescence (Haesebrouck and Pensaert 1986). The study conducted by Nayak et al. (1965) demonstrated that bronchial epithelial cells stained positive, demonstrating infection, within 2 hours of challenge. Within 4 hours, antigen was
detectable in the alveolar septa and numerous fluorescent cells were observed in the alveoli and alveolar ducts within 24 hours of infection. By 16 hours, there were large fluorescent areas of bronchial epithelium. This remained until 72 hours postinfection, after which the staining lessened. Fluorescent staining in both bronchioles and alveoli was absent by day 9 (Nayak et al. 1965).

2.6 Diagnosis

Infections with SIV can be diagnosed using a combination of clinical signs, typical gross lesions, histopathological lesions, isolation of the viruses and detection of virus-specific antibody. A presumptive diagnosis is based on clinical and histopathological findings. Diagnostic confirmation can be achieved by virus isolation or detection of SIV antibody (Merck & Co. 2006).

2.6.1 Clinical signs

Clinical signs and nasal shedding of SIVs can appear by 24 hours after infection. Virus shedding typically stops within day 7–10 of infection (OIE 2008). The typical acute outbreak is recognised by a rapid onset and spread through the entire herd, usually within 1 to 3 days. The primary signs comprise depression, fever (40.5-41.7°C), anorexia, coughing, dyspnea, weakness, prostration, and ocular and nasal discharges (Merck&Co. 2006). Loss of weight and weakness arise from anorexia and inactivity. Regarding economic losses, arresting of mean growth for 5 to 8 days and mean weight losses of 5 to 6 kg have been documented (Easterday and Van Reeth 1999). Morbidity is high (near 100%) but mortality is low (usually less than 1%) unless there are concurrent infections or the infected pigs are very young (Easterday and Van Reeth 1999). The course of the disease is usually 3-7 days in cases of
uncomplicated infection, with clinical recovery by 5-7 days after the onset of the disease.

Infections with SIV can affect reproductive performance in both males and females. In particular, the H3N2 strain in the USA has been involved in reproductive loss. Elevated body temperature in boars can cause adverse effects on spermatogenesis, leading to reduced fertility. In females, a variety of reproductive problems occur depending on the stage of gestation, including delayed return to estrus, abortions, decreased litter size and reduced viability of piglets at birth (Yoon et al. 2002). It is inconclusive whether SIVs can pass the placental barrier and infect foetuses. In addition, the direct role of SIVs in pregnancy wastage and infertility is not clear. Generally, it is believed that reproductive problems caused by SIV infections arise from high fever (Yoon et al. 2002). Aborting sows are usually “off feed” for a few days with a fever of ≥41°C. Abortions may be widespread, ranging from 5 to 10% of sows without immunity to SIVs. Abortion storms can resolve within 2 to 3 weeks (Yoon et al. 2002). Milk production in lactating females may decrease, resulting in adverse effects on nursing piglets. According to production records, it has been observed in the field that SIV infections increase the number of non-pregnant sows and the sow mortality rate (Yoon et al. 2002).

There are two clinical forms of SIV (epidemic or epizootic and endemic). Influenza epidemics can emerge if the viruses infect susceptible seronegative pigs or if the infection is intensified by factors such as poor husbandry, stress, secondary infections, cold weather and extreme temperature fluctuations. In the epidemic form, the onset of disease is acute and dramatic, particularly in the sow herd (Yoon et al.
The viruses spread quickly through all phases of a swine unit with rapid recovery if there are no complicating factors such as secondary bacterial infections. In contrast to the epidemic form, clinical signs in the endemic form may be less apparent and not all pigs may present typical clinical signs of infection (OIE 2008). Typical signs of influenza may occur in only 25 to 30% of pigs in the herd. Clinical disease is more obvious in a young pig herd. The clinical cases can be complicated by secondary infections with respiratory bacterial pathogens such as *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Haemophilus parasuis* and *Streptococcus suis* type 2 (Yoon et al. 2002). In pig herds with an acute or epidemic form, the disease usually becomes endemic subsequently unless proper management decisions and interventions are implemented (Yoon et al. 2002).

On the basis of clinical signs, pigs infected with one subtype of SIVs cannot be distinguished from pigs infected with other subtypes (Yoon et al. 2002). There are no differences in virulence between all three common virus subtypes: H1N1, H3N2 and H1N2 (Van Reeth 2007). However, based on field reports, when H3N2 subtype made its first appearance in the USA, the infections were more severe than H1N1 subtype infections. The severity of H3N2 SIV infection was observed by field observations, but isolates have not been evaluated for differences in virulence (Yoon et al. 2002). The probable reason may be due to the fact that many pigs were vaccinated with H1N1 SIV vaccines or pigs already had antibodies from natural infection with a H1N1 subtype; as a result, they were protected from severe clinical signs caused by H1N1 SIV infection. For H1N1 subtype, it is believed that the “avian variant” or “avian-like” virus circulating in Europe acquired a more
pathogenic attribute than the “classic” H1N1 virus circulating in the USA and other countries (Easterday and Van Reeth 1999).

Recently, a number of pig herds infected with the A(H1N1)pdm09 virus have been reported worldwide. It appears that this virus causes mild disease. Morbidity rates were reported to vary from less than 1% to as high as 90%, but little or no mortality has been documented (CFSPH 2009). In addition, no deaths in experimentally challenged pigs have been recorded (Itoh et al. 2009). The clinical presentations caused by the A(H1N1)pdm09 virus are similar to those caused by other SIVs such as coughing, nasal discharge, fever, weakness, decreased appetite, abortions and diarrhoea. The most prominent signs among experimentally challenged pigs are nasal discharge, sneezing and fever. The experimentally infected pigs developed clinical signs on day 3 after inoculation (Lange et al. 2009). However, in one study, miniature pigs presented with no signs although they shed the virus (Itoh et al. 2009). In mice, ferrets and nonhuman primates, the A(H1N1)pdm09 virus could cause more severe clinical signs or lung pathology than seasonal human H1N1 viruses (Itoh et al. 2009; Maines et al. 2009; Munster et al. 2009).

2.6.2 Lesions

2.6.2.1 Gross lesions

The lesions of SI induced by natural infection are commonly complicated with intercurrent bacterial infections. In uncomplicated cases, the gross lesions are similar to those of viral pneumonia where the apical and cardiac lobes of the lungs are mainly affected. In severe cases, more than half of the lungs may be changed. Macroscopically, infected lungs have a purple-red multifocal to coalescing
consolidation of predominantly cranio-ventral portions of the lung, recognised as a checquer-board lung (Vincent et al. 2008). A sharp line between the affected and normal lung tissue may occur and interlobular oedema may be evident. Blood-tinged and fibrinous exudate may present in airways with enlarged associated and bronchial mediastinal lymph nodes. Fibrinous pleuritis may also be seen (Nayak et al. 1965).

In the province of Quebec, Canada, a severe proliferative and necrotising pneumonia (PNP) in pigs has been observed (Morin et al. 1990). A confluent consolidation of the cranial and middle lobes and the lower half of the caudal lobes has been reported. The cause of PNP is still unclear (Easterday and Van Reeth 1999). The lobes were reported to be red or gray and "moist and meaty" with interlobular oedema. Some authors suggested that the causative virus isolated from the lungs of infected animals was a swine H1N1 distantly related to the contemporary H1N1 viruses circulating in North America. However, Bikour et al. (1994) identified the virus as a new strain isolated for the first time in the Canadian pig population, which was related to A/Sw/Hong Kong/76 H3N2 SIV.

In Great Britain, differences in lesion severity with the different influenza virus variants have been recognised. The pathogenicity of the avian H1N1 strain in Europe was higher compared to the classic H1N1 strain in the USA (Easterday and Van Reeth 1999). Classical H1N1 and H3N2 strains circulating since 1986 presented minimal gross lesions and a very mild interstitial pneumonitis in both natural and experimental cases (Done et al. 1994). However, infection with the more recent H1N1 variant demonstrated marked gross lesions and more severe histopathological
changes, including severe necrosis of bronchial epithelium, alveolar exudation and neutrophil infiltration (Easterday and Van Reeth 1999).

2.6.2.2 Microscopic lesions

The microscopic lesions in uncomplicated cases of SI are similar to those of viral pneumonia. There is widespread degeneration and necrosis of the bronchi and bronchioli epithelium. Exudates with desquamated cells and neutrophils (later mostly monocytes) fill the bronchi, bronchioli and alveoli lumen. Variable hyperaemia with dilatation of the capillaries and infiltration of the alveolar septae with lymphocytes, histiocytes and plasma cells can be observed and followed by widespread alveolar atelectasis, interstitial pneumonia and emphysema. Peribronchial and perivascular cellular infiltration can be seen (Easterday and Van Reeth 1999). The PNP histopathological lesions include exudative lesions with protein-rich edema and large macrophages in alveoli, necrotising bronchiolitis (mainly affecting terminal bronchioles), proliferative lesions marked by proliferation of type II pneumocytes and alveolar epithelialisation, coagulates of necrotic cells in alveolar ducts and alveoli, and hyaline membrane formation in the lumen of the terminal alveolar ducts. During recovery, bronchiolar epithelium is proliferative and lymphocytic cuffing becomes more apparent.

2.6.3 Virus isolation and detection of antigen

2.6.3.1 Identification of the agent

Sick pigs with clear nasal discharge and high fevers (≥41°C) should be chosen for sampling (Yoon et al. 2002). The most common samples used for diagnosis are fresh and formalin-fixed lungs collected at necropsy from pigs with respiratory illness.
Nasal or lung airway swab samples can be used for virus isolation or commercially available antigen-capture ELISA (Yoon et al. 2002). Viruses can be isolated in cell culture or in 9 to 10-day-old embryonated chicken eggs. Allantoic fluid is collected from these eggs and tested for haemagglutination after 72 hours of incubation (Easterday and Van Reeth 1999). Cell lines and primary cells susceptible to SIV infection can be used for cell culture, preferably Madin–Darby canine kidney (MDCK) cells. If cytopathic effects (CPE) are found in the cell culture during the incubation period (5-7 days), an aliquot of the cell culture medium may be tested for haemagglutinating viruses or by reverse transcription-polymerase chain reaction (RT-PCR) for conserved influenza virus genes such as nucleoprotein or matrix (OIE 2008). With detailed genomic analysis, sequencing can be conducted in order to further characterise a genetic component of virus isolates (Yoon et al. 2002).

### 2.6.3.2 Subtyping

For virus isolation, the subtype of influenza viruses must be determined to the H and N components. The standard protocol for subtyping these viruses includes the HI test and the neuraminidase inhibition test. However, these techniques require viruses isolated with relatively high HA titre (Yoon et al. 2002). With technological advances, molecular assays have been developed for subtyping. The PCR can be applied directly on clinical specimens without performing virus isolation. These assays provide a quick result and have high sensitivity. Multiplex format of PCR can identify coinfection of SIV with different subtypes in samples (Yoon et al. 2002).
2.6.4 Serology

The most common serological test for the detection of SIV antibodies is the HI test. The test is regarded as a moderately sensitive but highly specific test. The test results appear to have a good correlation with protective immunity (Yoon et al. 2002). In addition to the HI test, other serological tests occasionally used are the virus neutralisation, agar gel immunodiffusion test and the indirect fluorescent antibody test. ELISA commercial kits are also used and considered to be the most sensitive serological assays.

For the serological diagnosis of SI, paired serum samples collected 10–21 days apart should be conducted in order to demonstrate an increase of antibody when tested against appropriate antigens (Easterday and Van Reeth 1999; OIE 2008). The H1 and H3 assays must be used separately to detect both H1 and H3 antibodies (Yoon et al. 2002). Serum samples for serological tests should be collected at least 1 week after infection is suspected. Increasing antibody titres of 1:10 to 1:20 are considered as suspect and 1:40 or greater are considered as positive. Moderately high titres of 1:80 to 1:160 occur by 7 days after infection. Peak titres of 1:320 to 1:640 occur 10 to 14 days post-infection, although it may take as long as 3 weeks in some cases. The titres remain at fairly high levels until at least 4 weeks after infection before they start to decline (Yoon et al. 2002). Nevertheless, test results of the HI test must be interpreted with care as nonspecific inhibitors and nonspecific agglutinins may be present in pig sera that may interfere with the test results (Easterday and Van Reeth 1999). Moreover, weaner pigs with maternally-derived antibodies may be infected and may shed viruses. The positive results in suckling or weaner pigs from dams with antibody to the virus can thus be complicated (Easterday and Van Reeth 1999).
Longitudinal studies may be essential to monitor SIV in vaccinated herds (Yoon et al. 2002).

2.7 Treatment

For SI, there is no specific treatment. Infected animal are treated with supportive care. During the acute stages of the disease, pigs should not be moved or transported to avoid additional stress. It is very important to prepare comfortable, dry, dust-free bedding and draft-free and clean shelter. Since most animals become febrile, fresh and clean drinking water should be available at all times (Easterday and Van Reeth 1999). For herd treatments, expectorants are generally used and administered in drinking water. Generally, antibiotics and other antimicrobial treatments are used in order to control concurrent or secondary bacterial infections; however, antiviral drugs are not commonly provided to affected animals.

2.8 Prevention and Control

2.8.1 Biosecurity measures

Biosecurity practices should be implemented so as to prevent susceptible animals from having contact with infected animals. These practices consist of limiting the access of people and vehicles to the premises, proper cleaning and disinfecting of transport vehicles and quarantining new stock before introducing them to the pigs on a farm (Yoon et al. 2002). Sick animals should be identified and isolated as soon as possible. Given the possibility of interspecies transmission of influenza A viruses on pig farms, management practices should include measures that prevent pigs from contacting other species, particularly avian species. Moreover, humans suspected to
be infected with influenza virus should not be allowed to have contact with pigs on a farm (Easterday and Van Reeth 1999).

2.8.2 Vaccination Strategies

Vaccines are regarded as one of the most effective tools to prevent the spread of the influenza viruses and also to mitigate the severity of illness and the diseases impact (Girard et al. 2010). The vaccines help to induce high titres of IgG in sera and lungs in order to protect against clinical disease. In sows, vaccination is commonly adopted to protection against clinical disease. This also enhances and prolongs maternally-derived antibody levels to protect SI in young pigs (Thacker and Janke 2008). Following vaccination, protection of SIV infections depends upon the presence of HI antibodies in pig sera (Easterday and Van Reeth 1999). Pre-existing SIV HI antibodies must be <1:40 so as not to interfere with the action of the vaccines.

SI vaccines are adjuvanted and inactivated whole-virus vaccines prepared typically from the virus propagated in embryonated chicken eggs. Although these vaccines do not prevent infection or virus shedding, as the protection against infection is not complete, virus multiplication and shedding are considerably diminished when the disease occurs. In order to reduce reproductive losses, the vaccine can be administered in replacement gilts, boars and pregnant pigs before farrowing. Since maternal immunity from unvaccinated sows and vaccinated sows could last until 8 weeks and 20 weeks of age, respectively, producers should not vaccinate pigs before the age of 8 weeks to avoid interference by maternally-derived antibodies (Yoon et al. 2002).
Currently, available vaccines are autogenous or licensed vaccines. Generally, pigs are vaccinated intramuscularly with 2 injections 3 weeks apart. Commercial SIV vaccines contain potent oil-in-water adjuvants. The strain composition of the vaccines differs between Europe and the USA according to the antigenic and genetic differences of the predominant viruses (OFFLU 2010). In Europe, vaccines against SIVs are commonly used. There are commercially available inactivated whole-virus vaccines and split-product vaccines (Easterday and Van Reeth 1999). As the vaccines containing only one subtype do not protect against infection with other subtypes, vaccines are prepared from the H1N1 and H3N2 influenza virus strains of human origin in an oil-in-water adjuvant. The use on mainland Europe is localized and combined with vaccines against Actinobacillus pleuropneumoniae and Aujeszky's disease virus (Yoon et al. 2002).

In the USA, vaccination against SIVs is a common practice in the pig industry. Most fully licensed vaccines are multivalent in nature with various combinations of H1 and H3 subtypes (Yoon et al. 2002). Recently, autogenous vaccines have been increasingly used in the USA in order to cope with the periodical change of SIVs in nature. In 2008, over half of the doses of marketed SIV vaccine were autogenous and most of them were multivalent with H1 and H3 subtypes. The vaccines require only basic purity and safety testing, not the rigorous purity, safety, potency and efficacy testing that fully licensed SIV vaccines require. However, these vaccines can be distributed and used only in the herd of origin (Vincent et al. 2010).

Research on novel vaccines such as live-virus, vectored or DNA vaccines has been undertaken (Thacker and Janke 2008). To be specific the studies of vectored
vaccines using vaccinia virus, baculovirus, alphavirus or adenovirus have been reported (Wesley et al. 2004). Recently, DNA vaccines have been studied by using chicken, mouse, ferret and primate models of influenza virus infection. DNA vaccines provide heterosubtypic immunity and the internalisation of DNA inside host cells that would decrease interference by maternal immunity. The other advantages include the production of normal viral protein without the risks associated with the use of live viruses and the stimulation of long-lasting immunity through both humoral and cell-mediated immunity (Thacker and Janke 2008). Nevertheless, to date experimental trials of DNA vaccines in pigs have not been successful. More importantly, there is significant concern of using live-virus vaccines as new reassortant viruses between field strains and strains in the vaccines could result.

With respect to economic aspects, there are contradictory reports on the benefit of SI vaccination in the field. In commercial fattening herds, immunity induced by SI vaccination with inactivated vaccines may occur too late because SIV infections appear very soon after the entry of feeder pigs. In contrast in closed breeding-fattening farms in Belgium, vaccination of feeder pigs is considered cost-efficient, especially in winter when infections are more harmful (Easterday and Van Reeth 1999). Importantly, the development of a vaccine strain is essential for controlling SIVs and reducing the risk of reassortment with the current A(H1N1)pdm09 virus or other emerging viruses in the future (Vincent et al. 2010). Effective vaccination of pigs against SIV infection will decrease not only SI morbidity rate in pigs, but also the potential of pigs serving as reservoirs of influenza viruses for humans (Olsen et al. 2000).
There are a number of obstacles for successful vaccination against influenza in pigs. These comprise viral antigenic shift, antigenic drift and the effect of maternally-derived antibodies on vaccine efficacy. To illustrate, piglets are not vaccinated until their maternal immunity decays as vaccine efficacy can be reduced by maternally-derived antibodies. The piglets can therefore become susceptible to SIV infections prior to vaccination, leading to an increased incidence of the disease (Kitikoon et al. 2006). Moreover, regular and continual antigenic and genetic characterisations of SIVs are important to help veterinarians determine current antigenic variant strains circulating in the population. Influenza vaccines may thus change periodically to reflect the contemporary subtypes and strains in a geographic area (Heinen 2003).

2.8.3 Knowledge, Attitudes and Practices (KAPs)

In the context of public health, studies about knowledge, attitudes, and behaviours towards infectious diseases are crucial in order to support improved communication strategies and to reduce the spread of disease. Understanding the perceptions of the public and their potential resources helps identify knowledge gaps which may be utilized for developing educational programs so as to increase the awareness of the public to infectious disease threats (Balkhy et al. 2010). Importantly, it is necessary to ensure that the public is suitably informed on how to avoid or minimise exposure to infectious agents. For example, basic knowledge about disease transmission, availability of vaccines and effective medical treatment are essential during an outbreak of infectious disease. It has been demonstrated that increased communication between physicians and the public improves understanding about disease and helps disseminate accurate information about the role that the public can play in reducing disease spread (Balkhy et al. 2010).
Recently, the swine origin of the A(H1N1)pdm09 virus has highlighted the role of pig farmers in serving as a bridging population for interspecies transmission of influenza A viruses between pigs and humans (Gray et al. 2007). Given frequent exposure to pigs, farmers may introduce zoonotic influenza virus into their households and communities that could increase the risk of emergence of a novel influenza virus strain with pandemic potential (Gray et al. 2007). Thus, understanding KAPs of pig farmers is vital for development of a communication plan, through collaboration between the public health and animal health sectors, to prevent and control interspecies influenza epidemics. During poultry outbreaks of avian influenza H5N1 studies were conducted regarding levels of knowledge, attitudes, and practices towards avian influenza in high-risk populations in Thailand (Olsen et al. 2005; Maton et al. 2007). However to the best of our knowledge, there has been nothing in the literature to date describing KAPs for Thai pig farmers and swine influenza.

2.9 Surveillance programs

2.9.1 Swine influenza viruses

Disease surveillance schemes should adjust to different pig production systems, particularly backyard and small pig producers in developing countries. Surveillance at this level should consist of the active participation of local communities and farmers to report respiratory cases in pigs. Pigs with clinical signs, such as fever, anorexia and laboured abdominal breathing, and suggestive post-mortem findings of SI can be potential candidates for surveillance sampling (OFFLU 2010). Serological data may be a useful screening tool to target more intensive viral surveillance (Ciacci-Zanella et al. 2010). Serological studies, however, cannot differentiate
between the A(H1N1)pdm09 virus and other strains of influenza A H1 circulating in pigs due to cross-reactivity (Kyriakis et al. 2010). Therefore, virological testing is necessary to characterise the viruses of pigs. Moreover, virological and epidemiological data between animal and human health sectors enhances the understanding of influenza A viruses in pigs and their risks that could lead to an early warning to emerging threats (OFFLU 2010).

2.9.2 The A(H1N1)pdm09 virus

Recently, a number of countries have begun routine surveillance to detect the occurrence of the A(H1N1)pdm09 virus and to perform regular surveillance activities for other respiratory syndromes e.g. porcine reproductive and respiratory syndrome (PRRS) (Ferrari et al. 2009). Since infections of the A(H1N1)pdm09 virus in pigs have not caused a significant animal health problem, the main objective of surveillance for influenza A viruses in pigs is to protect public health. To illustrate, virological surveillance could identify reassortment events or genetic mutations that could assist in pandemic preparedness planning (OFFLU/WHO 2009). Thus, virological surveillance for the A(H1N1)pdm09 virus is the most preferable, but it must be conducted with the correct protocols and expertise. Molecular assays for virus isolation are the most sensitive and specific techniques for detection of the A(H1N1)pdm09 virus in pigs (OFFLU/WHO 2009). When designing a surveillance plan, the national laboratory testing capacity should be capable of undertaking the necessary level of testing (Ferrari et al. 2009). In particular, the OIE/FAO animal influenza network of international reference laboratories (OFFLU 2010) offers a list
of reference laboratories, a testing algorithm and technical recommendations for sample collection and shipment (Ferrari et al. 2009).

Thereafter, reassortment events of the A(H1N1)pdm09 viruses and other endemic swine influenza viruses have been reported, suggesting the establishment of the A(H1N1)pdm09 virus in the Thai pig population (Kitikoon et al. 2011a; Hiromoto et al. 2012). Following the introduction of the A(H1N1)pdm09 virus, surveillance for influenza viruses was conducted in the Thai pig population from 2010 to 2012 (Charoenvisal et al. 2013). It was found that 23 viruses were isolated from 1,335 samples in the study (1.72%). The viruses consisted of the A(H1N1)pdm09 virus (7 isolates, 0.52%), reassortant A(H1N1)pdm09 virus (1 isolate, 0.07%), Thai endemic H1N1 virus (3 isolates, 0.22%), reassortant H3N2 virus with A(H1N1)pdm09 internal genes (9 isolates, 0.67%) and reassortant H1N2 virus with A(H1N1)pdm09 internal genes (3 isolates, 0.22%) (Charoenvisal et al. 2013). This has emphasised the genetic diversity of influenza A viruses in pigs in Thailand. In the following chapter virological surveillance for influenza viruses in pigs was conducted at 5 pig farms in Chonburi and Chachoengsao provinces, Thailand to monitor the genetic characteristics of influenza viruses and to allow the early detection of novel viruses with pandemic potential.
Chapter 3: Detection of influenza A viruses in pigs from commercial farms in Thailand

3.1 Introduction

Influenza A viruses are enveloped, single stranded RNA viruses belonging to the family Orthomyxoviridae, which contains 4 other genera: influenza B virus, influenza C virus, thogotovirus and isavirus (Ma et al. 2009). The genome of influenza A viruses contains 8 negative sense segments, encoding 10 recognised gene products including PB1, PB2 and PA polymerases, HA, NP, NA, M1 and M2 proteins, and NS1 and NS2 proteins (Webster et al. 1992). Whilst two or more influenza strains concurrently infect a cell, the segments from different strains can be mixed and rearranged in the progeny viruses (Webster et al. 1992), potentially leading to reassortment events in a host. Given the pig trachea contains the sialic acid receptors preferred by both avian and human influenza viruses (Ito et al. 1998), pigs have been proposed to act as intermediate hosts or “mixing vessels” of influenza viruses from different sources (Landolt and Olsen 2007). If pigs are co-infected with avian influenza viruses and another strain such as a human or swine virus, a reassortant virus could be generated and established in pigs. This may explain why pigs have often been implicated in the emergence of influenza pandemics in human populations (Ma et al. 2009).
In the 2009 pandemic, the causative A(H1N1)pdm09 virus was generated by a reassortment event of two SIVs, namely Eurasian and North American triple-reassortant viruses (Garten et al. 2009). The former contributed the N1 and M segments and the latter provided the PB2, PB1, PA, H1, NP and NS segments (Garten et al. 2009). Since its appearance in humans, the A(H1N1)pdm09 virus has been introduced into pigs and other domestic animal populations worldwide (Howden et al. 2009; Mathieu et al. 2010; Welsh et al. 2010; Howard et al. 2011; Nfon et al. 2011; Lin et al. 2012). Later, reassortants of the A(H1N1)pdm09 virus and the pre-existing SIVs have been identified across the world. In China, reassortants of H1N1 (Vijaykrishna et al. 2010) and H3N2 subtypes (Fan et al. 2012) have been reported. In the USA, multiple reassortments of H1N1 and H3N2 subtypes have also been confirmed (Ducatez et al. 2011; Liu et al. 2012a). An H3N2 reassortant between a triple-reassortant SIV and the A(H1N1)pdm09 virus was recognised in Canada and the virus was also isolated from mink (Tremblay et al. 2011). In Europe, reassortants of the H1N1 and H1N2 subtypes have been found in Germany, Italy and UK (Howard et al. 2011; Moreno et al. 2011; Starick et al. 2011).

In Thailand, H1N1, H3N2 and H1N2 SIV subtypes have been circulating in pig populations for several decades (Sreta et al. 2010). Historically, the H3N2 SIV subtype was firstly isolated in 1978 (Nerome et al. 1981). Later in 1988, the classical H1N1 SIV was identified (Kupradinun et al. 1991). In 2005, the emergence of a new subtype H1N2 was reported (Damrongwatanapokin et al. 2006). Phylogenetic analysis of Thai SIVs suggested the presence of genetic diversity and there have been multiple introductions of classical swine, avian-like swine and human influenza viruses into the Thai pig population (Takemae et al. 2008). After the emergence of
the A(H1N1)pdm09 virus in Thais, the virus was subsequently isolated from pigs in Thailand (Sreta et al. 2010; Takemae et al. 2011) and was followed by reassortment between the A(H1N1)pdm09 virus and Thai SIV (Kitikoon et al. 2011a). Therefore, virological surveillance for influenza A viruses in Thai pig population should be carried out to monitor the genetic characteristics of influenza viruses and to allow the early detection of novel viruses with pandemic potential. In this study, virological surveillance for influenza viruses in pigs was conducted at 5 pig farms in Chonburi and Chachoengsao provinces, Thailand in February 2011.

### 3.2 Materials and methods

#### 3.2.1 Farm selection

Virological surveillance for influenza viruses in pigs was carried out in Chonburi and Chachoengsao provinces (Figure 3.1) that have a high density of commercial pig farms. Five commercial pig farms with farrow-to-finish operations were recruited by provincial veterinary officers, based on the farmer’s willingness to cooperate. All pig farmers provided informed consent.
Figure 3.1. Map of Thailand showing the location of the provinces included in the study.

3.2.2 Sample collection

Sixty nasal swabs were collected from each farm (from 20 sows, 20 weaners and 20 fattening pigs). Sows were chosen using the random number function in Microsoft
Excel 2007. As weaners and fattening pigs had no identification numbers, samples were collected from pig groups by random sampling of pig pens. Pens containing weaners or fattening pigs were assigned a number and then those to be sampled were selected using the random number generator in Microsoft Excel 2007. Then, 20 samples were collected from each of the selected pens. This resulted in a total of 300 swabs collected from 5 farms.

During the visit, information on the management and husbandry practices adopted by the farm were collected along with the identification, sex and age of the sampled pigs. All samples were collected from clinically normal pigs at the time of sampling and none showed any signs of respiratory illness. Sampled pigs were restrained by a snare following standard procedures. A sterile swab (Denka Seiken Co., Ltd., Tokyo, Japan) was inserted deeply into one nostril (Figure 3.2). The tip of the swab was placed into a plastic vial containing 2–3 ml of virus transport medium and the applicator stick was broken off. The vials were labeled with a specific code containing the farm identification number and nasal swab sample number. The specimens were stored in a styrofoam container with ice packs and rapidly transported to the laboratory at the National Institute of Animal Health (NIAH) in Bangkok. All specimens were processed within the day of sampling.
3.2.3 Virus isolation

Before virus isolation, a portion of each of five nasal swab samples was pooled and pools were tested by M gene real-time polymerase chain reaction (PCR) for detection of influenza A viruses. Briefly, 40 µl from each of 5 individual swab specimens with successive sample numbers were pooled to 200 µl. This resulted in 60 pooled samples. RNA was extracted from the pooled swab samples using the RNeasy Mini Kit (Qiagen, Inc. GmbH, Hilden, Germany). The extracted RNA was reverse-transcribed to cDNA using Superscript III (Invitrogen, Carlsbad, CA, USA) with a universal primer for influenza A viruses (Hoffmann et al. 2001). The resulting cDNA was tested by SYBR green real time PCR using SYBR® Premix Ex TaqTM (Takara Bio Inc., Shiga, Japan) with primers targeting the M gene of the influenza A
viruses. The primers used consisted of M33F (5’-TTCTAACCAGGTGAAACG-3’) and M264R2 (5’-ACAAAGCGTCTACGCTGCAG-3’) (Ngo et al. 2012).

Only individual swab samples from the pools that tested positive by M gene real-time PCR were subjected to virus isolation. The swab specimens were filtered with a 0.45 µm pore size filter (Millipore, MA, USA) and were inoculated onto monolayers of MDCK cells and into the allantoic cavities of 10-day-old embryonated chicken eggs. If cytopathogenic effects were observed within 4 days, the supernatant was tested for influenza viral antigens with a rapid diagnostic kit, namely QuickNavi™ (Denka Seiken Co., Ltd., Tokyo, Japan). For each sample, the supernatant was harvested and tested by the haemagglutination test with 0.5% guinea pig red blood cells. Subtypes of the HA and NA genes were determined by RT-PCR with subtype-specific primers designed in a previous study by Takemae et al. (2008).

3.2.4 Sequence and phylogenetic analysis

Viral RNA and cDNA of the isolated viruses were extracted and synthesised as described above. Each gene segment was amplified by PCR with Ex Taq (Takara Bio Inc., Shiga, Japan) and segment specific primers (Takemae et al. 2008). The PCR products were purified with the QIAquick Gel extraction kit (Qiagen GmbH, Hilden, Germany) and were sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer (Life Technologies, Carlsbad, California, USA). Phylogenetic trees were constructed using the MEGA5.2 software with 1,000 bootstrap replicates of the neighbour-joining method. The
sequences determined in this study are available from GenBank under accession number AB704796–AB704859.

3.3 Results

3.3.1 Farm information

Information on the five farms sampled is summarised in Table 3.1. The farms comprised three farms in Chonburi province and two in Chachoengsao province. Four farms used an evaporative cooling system (EVAP), Farm A had open housing allowing natural air ventilation. All farms were large-scale commercial farms, housing between 4,000 and 20,000 pigs. Farms A, D and E introduced breeders from local pig breeding farms, while farms B and C were closed herds. Raising other animals in pig farms were observed on farms A, D and E. Vaccination was applied in all farms (see Table 3.1 for specific vaccination details).
Table 3.1. Basic information on the sampled farms in Chonburi and Chachoengsao provinces, Thailand.

<table>
<thead>
<tr>
<th>Farm</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Province</td>
<td>Chonburi</td>
<td>Chonburi</td>
<td>Chonburi</td>
<td>Chachoengsao</td>
<td>Chachoengsao</td>
</tr>
<tr>
<td>Type of operation</td>
<td>Farrow to finish</td>
<td>Farrow to finish</td>
<td>Farrow to finish</td>
<td>Farrow to finish</td>
<td>Farrow to finish</td>
</tr>
<tr>
<td>Ventilation system</td>
<td>Open</td>
<td>EVAP</td>
<td>EVAP</td>
<td>EVAP</td>
<td>EVAP</td>
</tr>
<tr>
<td>Total number of pigs</td>
<td>4,000</td>
<td>20,000</td>
<td>11,000</td>
<td>9,000</td>
<td>14,000</td>
</tr>
<tr>
<td>Sources of breeders</td>
<td>Domestic farm</td>
<td>Homebred</td>
<td>Homebred</td>
<td>Domestic farm</td>
<td>Domestic farm</td>
</tr>
<tr>
<td>Presence of other animals</td>
<td>Chickens, Dog</td>
<td>-</td>
<td>-</td>
<td>Cattle, Chickens, Dog</td>
<td>Cat, Dog, Fish</td>
</tr>
<tr>
<td>Vaccination against:</td>
<td>FMD, PRRS</td>
<td>AD, FMD, PRRS, CSF</td>
<td>AD, Circovirus, FMD, PED, PEL, PRRS, MP, CSF</td>
<td>AD, Circovirus, FMD, CSF</td>
<td>AD, AR, Circovirus, FMD, Parvovirus, CSF</td>
</tr>
</tbody>
</table>

\*Foot and mouth disease  
\*Porcine Reproductive and Respiratory syndrome  
\*Aujeszky's disease  
\*Classical Swine fever  
\*Porcine Epidemic Diarrhoea  
\*Parvovirus-Erysipelas-Leptospirosis  
\*Mycoplasma

### 3.3.2 Virus isolation

Two pooled samples from farm B in Chonburi province were positive for the influenza virus M gene by real-time PCR. From one of the two pools, two individual swab samples yielded H3N2 viruses only from the MDCK cells. Haemagglutination titres reached 16–32 after two passages. The isolates were designated as

From farm D in Chachoengsao province, two pools were positive for the influenza virus M gene by real-time PCR. Three viruses from one of those pools were isolated from both the MDCK cells and embryonated eggs and one virus was isolated only from the eggs. Two individual swabs from the other positive pool yielded viruses in the MDCK cells and eggs. Virus titres in the first or second passages in the MDCK cells were 1–2 HA units, while those in the eggs were HA units of 8 or higher. Those isolates were designated as

A/swine/Chachoengsao/NIAH107037-21/2011,
A/swine/Chachoengsao/NIAH107037-22/2011,
A/swine/Chachoengsao/NIAH107037-23/2011,
A/swine/Chachoengsao/NIAH107037-24/2011,
A/swine/Chachoengsao/NIAH107037-28/2011

and A/swine/Chachoengsao/NIAH107037-29/2011.

3.3.3 Sequence and phylogenetic analysis

The sequences of the full-length protein coding regions of the Chonburi and Chachoengsao egg isolates were determined. BLAST analysis revealed that the HA and NA genes of the Chonburi isolates were closely related to the seasonal H3N2 human virus isolated in 1996, while 6 internal genes showed the highest homology (99%) to the A(H1N1)pdm09 virus (http://blast.ncbi.nlm.nih.gov/, accessed on 10th February 2012). All gene segments of the Chachoengsao isolates showed the highest homology (99%) to the A(H1N1)pdm09 virus (Table 3.2). Nucleotide identity
among the Chonburi isolates ranged from 99.9% to 100% and that of the Chachoengsao isolates from 99.1 to 100%. Nucleotide identities between the Chonburi and Chachoengsao isolates were 99.1–99.2% for the PB2, 99.1–99.2% for the PA, 99.1% for the M, 96.3–96.6% for the PB1, 97.2–97.5% for the NP and 95.5–96.1% for the NS genes. To reveal the genetic origin of the swine H3N2 influenza virus in this study, phylogenic trees of HA and NA segments were constructed. The H3 HA gene of the H3N2 isolates belonged to cluster Ha of the Thai SIVs (Takemae et al. 2008) (Figure 3.3). Similar topology could be seen in the tree of the N2 NA gene (Figure 3.4).
Table 3.2. Swine influenza virus strains isolated in Thailand with segments homologous to the 2009 pandemic A (H1N1) virusa.

<table>
<thead>
<tr>
<th>Province</th>
<th>Strain</th>
<th>Subtype</th>
<th>% Homology (accession number)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PB2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PB1</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>PA</td>
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<td></td>
<td></td>
<td></td>
<td>HA</td>
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<td></td>
<td></td>
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<td>M</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Chonburi</td>
<td>A/swine/Chonburi/NIAH106952-026/2011</td>
<td>H3N2</td>
<td>99</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(AB704796)</td>
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<td>(AB704803)</td>
</tr>
<tr>
<td>Chonburi</td>
<td>A/swine/Chonburi/NIAH106952-028/2011</td>
<td>H3N2</td>
<td>99</td>
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<tr>
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<td>(AB704804)</td>
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*Homology analysis was performed with genome segments of A/California/04/2009 (H1N1) (GenBank FJ966079–FJ966086).
Figure 3.3. Phylogenetic tree based on the nucleotide sequences from the H3 gene of Thai isolates compared to the selected seasonal human H3N2 viruses available from GenBank. Scale bars indicate substitution per site. Viruses isolated in this study and Thai SIVs are indicated by a dark triangular and circle, respectively.
Figure 3.4. Phylogenetic tree based on the nucleotide sequences from the N2 gene of Thai isolates compared to the selected seasonal human H3N2 viruses available from GenBank. Scale bars indicate substitution per site. Viruses isolated in this study and Thai SIVs are indicated by a dark triangular and circle, respectively.
3.4 Discussion

Two swine H3N2 influenza viruses isolated from weaning pigs from Farms B and D in Chonburi and Chachoengsao provinces were reassortant viruses consisting of the HA and NA genes from a swine influenza virus previously circulating in the Thai pig population and other internal genes from the A(H1N1)pdm09 virus. Despite the different origins of the HA and NA genes, H3N2 SIVs possessing internal genes from A(H1N1)pdm09 viruses have also been isolated in Canada and China. The HA and NA genes of the Canadian isolate were closely related to those of the triple reassortant virus originating from the seasonal human virus isolated around 1995–1997 (Webby et al. 2000). The HA and NA genes of the Chinese isolate were closely related to those of the Vietnamese triple reassortant SIV that had originated from the seasonal human virus isolated around 2004–2006 (Fan et al. 2012; Ngo et al. 2012). In the USA, H3N2 reassortant SIVs were also isolated from 2009 to 2011. They were found to have the HA and NA genes from the North American H3N2 human lineage and at least one other gene, the M gene, and up to five internal genes from the A(H1N1)pdm09 virus (Liu et al. 2012a).

The H3N2 reassortants and the A(H1N1)pdm09 viruses recovered in the current study were isolated from clinically healthy weaner pigs. The lack of clinical signs may be due to the presence of antibodies conferred through ingestion of colostrum (Corzo et al. 2013). Maternally derived antibodies could protect piglets against the clinical effects of influenza infections and piglets with maternal immunity may not show apparent clinical signs even though they may be shedding virus (Loeffen et al. 2003). Furthermore, low levels of exposure to virus might preclude clinical signs in
pigs (Van Reeth et al. 1999; Van Reeth 2000). It is important to note that subclinical infections in pigs have public health implications as humans may come in contact with clinically healthy pigs and could become infected if the pigs are shedding sufficient infectious viral particles (Gray et al. 2007; Killian et al. 2013).

Sporadic cases of zoonotic SIV infections have been confirmed worldwide including Europe, Asia and North America (Van Reeth 2007). Fifty cases of human infections with SIVs were documented during the period 1958–2005 (Myers et al. 2007). In the USA, 35 SIV human infection cases were confirmed from 2005 to 2011 (20 of subtype H3N2, 13 of subtype H1N1 and 2 of subtype H1N2) (Shinde et al. 2009). Recently, a new reassortant of swine H3N2 virus, with the M gene from the A(H1N1)pdm09 virus, caused human infections in the USA (Wong et al. 2012). These cases have underlined the importance of surveillance for influenza viruses in pigs, some of which may have the capacity to pose threats to public health.

In Thailand, human infection with SIV has been confirmed. In July 2005, an influenza virus, namely A/Thailand/271/2005 (H1N1), was isolated by the WHO National Influenza Centre, Bangkok from a 4-year-old male with influenza-like illness. The virus possessed HA and NS genes from classical swine and other genes from the Eurasian avian-like swine virus (Komadina et al. 2007). The patient recovered without further complications. No other cases of swine influenza were identified in humans at a similar time in these locations, suggesting that there was no extensive human-to-human transmission. Serological analysis also demonstrated evidence of pig-to-human influenza virus transmission in healthy workers from two
commercial pig farms in Thailand who were seropositive to Thai H1N1 and H1N2 isolates (Kitikoon et al. 2011).

In this study, the novel H3N2 reassortants and the A(H1N1)pdm09 virus strain isolated suggests that the A(H1N1)pdm09 virus has become established in the Thai pig population and consequently has resulted in genetic reassortment events with endemic SIVs in the country. Continuous surveillance for influenza A viruses in the Thai pig population is therefore essential to study virus evolution and to improve the understanding of virus ecology to provide benefits to the pig industry and public health. However, the chance of detecting SIVs is probably low owing to the usually short period of virus shedding in pigs. In previous studies, the detection rates of SIVs from pigs by virus isolation or real-time RT-PCR on individual nasal swabs were less than 5% (Wright et al. 1992; Peiris et al. 2001; Shieh et al. 2008; Trevennec et al. 2011; Corzo et al. 2013). This was also the case in the present study (2.7%, 95%CI: 1.2-5.2). Consequently, a large number of samples are required in order to successfully detect influenza A viruses in pigs (Trevennec et al. 2011). Where technical resources are limited, sampling strategies need to be refined in order to increase the possibility of isolating the virus and to maximise cost-effectiveness (as described in Chapter 5).

In the next chapter findings are reported from studies conducted into the potential risks for dissemination of influenza A viruses in and between smallholder pig farms in a selected rural community, where biosecurity may be suboptimal due to financial constraints. Questionnaire interviews were conducted to identify the risks of virus transmission arising from farmers’ and traders’ practices.
Chapter 4: Assessing Potential Risks of Influenza

A Virus Transmission at the Pig–Human Interface in Thai Small Pig Farms

4.1 Introduction

The epidemic of avian influenza A (H5N1) viruses in Asia, Africa and Eastern Europe and the 2009 influenza pandemic have demonstrated that influenza A viruses are a major public health threat worldwide (McCune et al. 2012). The influenza A (H1N1) virus responsible for the 1918 ‘Spanish flu’ pandemic emerged in humans and also established itself among pig populations as the ‘classical’ SIV (Crosby 1989). Similar to the 1918 pandemic, in 2009, soon after the A(H1N1)pdm09 virus was isolated from humans, it spread to pig populations throughout the world. Furthermore, it has since reassorted with endemic SIVs, and some of the resultant viruses have been shown to readily infect humans (Peiris et al. 2012). That has emphasised the complexity of the epidemiology and evolution of influenza viruses in both pig and human populations.

In cases of human infection with SIVs documented in the USA, Canada, Europe and Asia, most patients had a history of contact with pigs (Myers et al. 2007). If the first infection with a novel reassortant virus occurred in a pig herd, pig farmers could be among the first people to be infected and may serve as a source of virus for their communities (Saenzet al. 2006). In particular, pig farmers undertaking small-scale
pig production represent a high-risk population and need to be recognised as key actors in preventing cross-species pathogen transfer (McCune et al. 2012).

In Thailand, influenza A virus subtypes of H1N1, H3N2 and H1N2 have been found in pig populations for decades. Evidence of virus transmissions between pigs and humans has been reported with a swine-like influenza virus being isolated from a Thai patient (Komaladina et al. 2007). The findings of pig-to-human influenza virus transmission were also confirmed among pig farm workers in Thailand (Kitikoon et al. 2011). Conversely, the studies by Takemae et al. (2008) and Lekcharoensuk et al. (2010) suggested that the Thai H3N2 SIVs acquired the HA gene through the introduction of human H3N2 virus into the Thai pig populations. Moreover, after the emergence of the A(H1N1)pdm09 virus in humans, the virus and its reassortants have continued to be isolated from Thai pigs (Hiromoto et al. 2012).

In the context of global concern about a public health threat from influenza A viruses, knowledge of inter-species contact on pig farms, biosecurity practices adopted by farmers and the influence of pig-marketing chains is crucial, particularly in rural communities where biosecurity is often suboptimal. Evaluation of the risk of spread of influenza A viruses at the pig–human interface allows development of improved measures to reduce these risks. The aim of this study was to qualitatively evaluate the risks arising from farming practices of pig farmers and from trading activities of pig traders.
4.2 Materials and methods

The study was conducted in Mukdahan, a rural province in the north-eastern part of Thailand (Figure 4.1). It is considered a priority area to study as local smallholders usually raise domestic pigs that cohabit with free-range chickens.

Figure 4.1. Map of Thailand showing Mukdahan Province.
Structured questionnaires (Appendix 1 and 2) for face-to-face interviews were developed. These were designed to identify and describe the practices among farmers and traders and the interactions between humans and pigs that could be involved in the transmission of influenza A viruses. The questionnaire was approved by the Murdoch University Human Ethics Committee (Project no. 2012/121). A two-stage, household-based cluster sampling technique was employed. The first stage was selecting Wanyai district in Mukdahan based on its high density of small pig farms. The second stage consisted of selecting four of the 53 subdistricts in Wanyai district because these were the ones located close to Kaeng-Kabao where a high number of pig smallholders are located that provide pig products for tourists. Once subdistricts had been selected, a list of households owning livestock was developed in consultation with the local livestock officer appointed to each subdistrict. All households were then contacted and all agreed to participate in the study.

Participating pig farmers and traders were recruited through Wanyai livestock officers. Before conducting interviews, 20 livestock service providers (paravets) from the Wanyai livestock office were trained in the use of the questionnaires and questionnaires were assessed to ensure their comprehensibility, practicability and validity. Ninety-eight farmers and five traders were visited at home by the trained paravets. Verbal consent was obtained from all participants. Descriptive analyses of the results were carried out using SPSS version 17 (SPSS Inc., Chicago, IL, USA), including percentages for categorical data and means, SE and ranges for continuous data. The 95% confidence intervals for the percentage were calculated based on the exact binomial method (Ross, 2003).
4.3 Results
Interviewed pig farmers in the study were predominantly male (67.7%, 95%CI: 57.4–76.9). Sixty nine per cent of farmers had graduated from primary school (95%CI: 59.3–78.3). More than half of them had less than 3 years of pig-farming experience (58.2%, 95%CI: 47.8–68.1). Generally, participants practiced mixed farming, with rice growing as a principal component (77.6%, 95%CI: 68.0–85.4) and pigs providing additional cash income (97.9%, 95%CI: 92.7–99.7). In this area, the type of pig production was predominantly farrowing-to-weaning and most pigs were a Thai indigenous breed. The average number of pigs per farm was seven. Raising pigs together with chickens was commonly seen on the interviewed farms (93.6%, 95%CI: 85.7–97.9). Other animals present on the farms included cattle, dogs and cats (see Table 4.1).

Table 4.1. Type of animals raised in the surveyed households.

<table>
<thead>
<tr>
<th>Type of animals</th>
<th>No. of households (%)</th>
<th>No. of animals (% of all animals)</th>
<th>Mean</th>
<th>SE</th>
<th>Range</th>
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</thead>
<tbody>
<tr>
<td>Pigs</td>
<td>81 (100.0)</td>
<td>589 (23.9)</td>
<td>7.3</td>
<td>1.3</td>
<td>1 to 90</td>
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<tr>
<td>Chickens</td>
<td>73 (93.6)</td>
<td>1518 (61.5)</td>
<td>19.5</td>
<td>2.8</td>
<td>0 to 200</td>
</tr>
<tr>
<td>Cattle</td>
<td>35 (44.9)</td>
<td>109 (4.4)</td>
<td>1.4</td>
<td>0.2</td>
<td>0 to 9</td>
</tr>
<tr>
<td>Dogs</td>
<td>47 (60.3)</td>
<td>85 (3.4)</td>
<td>1.1</td>
<td>0.1</td>
<td>0 to 4</td>
</tr>
<tr>
<td>Cats</td>
<td>14 (18.2)</td>
<td>22 (0.9)</td>
<td>0.3</td>
<td>0.1</td>
<td>0 to 3</td>
</tr>
<tr>
<td>Other</td>
<td>15 (19.7)</td>
<td>146 (5.9)</td>
<td>1.9</td>
<td>0.7</td>
<td>0 to 30</td>
</tr>
<tr>
<td>Total</td>
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<td>2469</td>
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</table>

A number of husbandry characteristics that may increase the potential for virus spread on farms were observed. Firstly, utilising household waste for pig raising, for example swill (59.4%, 95%CI: 48.9–69.3), and pig manure for crop production
(78.7%, 95% CI: 69.1–86.5). Secondly, most pig production took place in conventional sties with wooden walls (93.5%, 95% CI: 86.5–97.6) and roofs made of leaves or metal sheets (87.6%, 95% CI: 79.4–93.4). Twenty-four per cent of farms were constructed so that piglets could escape and roam freely (95% CI: 15.4–34.1).

Thirdly, pigs were mostly raised on soil (54.8%, 95% CI: 44.2–65.2) and excessive water from rain could enter most pens (85.5%, 95% CI: 75.0–92.8). Fourthly, sweeping was the most common method of cleaning (84.9%, 95% CI: 76.0–91.5). Disinfectants were rarely used (8.6%, 95% CI: 3.8–16.2). Lastly, farmers rarely identified individual pigs by tattoo, ear notching or ear tags (see Figure 4.2 for details). Farmers usually recognised their pigs by their physical attributes (92.3%, 95% CI: 84.8–96.9).

![Figure 4.2. Bar chart presenting the percentage of different identification methods adopted for sows.](image-url)
Biosecurity risks that could facilitate spread of viruses were identified. Quarantine procedures were not applied in any of the studied farms. Hiring boars from other farmers for breeding was commonly adopted (66.7%, 95%CI: 55.5–76.6). Almost half of the farms allowed vehicles to drive close to the pens (49.5%, 95%CI: 38.9–60.0) and most farms had no on-farm policies for visitors, such as regulations on cleaning hands or shoes (91.6%, 95%CI: 84.1–96.3). Although most farmers wore protective boots (77.9%, 95%CI: 68.2–85.8), <10% of farmers wore gloves and masks (9.5%, 95%CI: 4.4–17.2 and 6.3%, 95%CI: 2.4–13.2, respectively) and 22% did not use any form of protective clothing on farm (95%CI: 14.2–31.8). Generally, they fed pigs twice a day and spent approximately 30 min each time with their pigs. Eighty-two per cent of farmers frequently cleaned their hands after touching their pigs (95%CI: 68.6–91.4). Washing hands with soap was common among farmers (94.0%, 95%CI: 83.5–98.7). Most farmers had never been vaccinated against the A(H1N1)pdm09 virus (93.2%, 95%CI: 85.7–97.5). When they were sick, 26% of them said they would continue to work on their farms (95%CI: 11.9–44.6).

Due to the potential for disease dissemination and threats to public health, biosecurity risks posed by the market chain were studied. Traders bought pigs at farms within their subdistrict more commonly than from different provinces (65%, 95%CI: 40.8–84.6 and 5%, 95%CI: 0.1–24.9, respectively). After traders purchased pigs from farms, they would commonly transport the live pigs by motorcycles (31.0%, 95%CI: 17.6–47.1) or pick-up trucks (66.7%, 95%CI: 50.5–80.4) to restaurants at Kaeng-Kabao. The volume traded per time varied from 20 to 100 head. As the price increased during the New Year and Thai traditional New Year festivals, higher numbers of pigs were sold at those times.
Regarding biosecurity practices, the risk was heightened by the fact that vehicles were rarely cleaned and disinfected. More importantly, if pigs were not delivered to the customers on the day of purchase, they were usually stored by traders on their own farms or at collection yards. The possible pathways for the inter-species transmission of influenza A viruses is illustrated in Figure 4.3.
Figure 4.3. Potential pathways of spread of influenza A viruses onto pig farms.
Potential modes of influenza transmission vary depending on the farmers’ practices on-farm and the traders’ behaviours during their trading activities. The questionnaire surveys revealed that most pigs had not been vaccinated (85.2%, 95%CI: 76.1–91.9). The most common clinical sign observed in pigs was diarrhoea (32.6%, 95%CI: 23.2–43.2). Influenza-like signs were also reported, including fever (8.7%, 95%CI: 3.8–16.4), cough or sneezing (7.6%, 95%CI: 3.1–15.1) and rhinorrhea (4.3%, 95%CI: 1.2–10.8). Farmers believed that pigs were more likely to be sick during the hot (April and June) and rainy seasons (August). When farmers found sick pigs, most would contact someone to get help, and in most cases, they contacted a paravet (see Table 4.2 for details). When pigs died, most farmers buried the body of the dead pig (78.6%, 95%CI: 69.1-86.2).

**Table 4.2. Actions taken by farmers when they found sick pigs.**

<table>
<thead>
<tr>
<th>Action</th>
<th>No. of households (%)</th>
<th>95% CI</th>
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<tbody>
<tr>
<td>Do nothing</td>
<td>1 (1.3)</td>
<td>0-6.9</td>
</tr>
<tr>
<td>Isolate sick pigs from others</td>
<td>32 (41.0)</td>
<td>30.0-52.7</td>
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<tr>
<td>Treat sick pigs themselves</td>
<td>38 (48.7)</td>
<td>37.2-60.3</td>
</tr>
<tr>
<td>Sell sick pigs</td>
<td>0 (0)</td>
<td>0-4.6</td>
</tr>
<tr>
<td>Slaughter for home consumption</td>
<td>0 (0)</td>
<td>0-4.6</td>
</tr>
<tr>
<td>Slaughter for selling</td>
<td>0 (0)</td>
<td>0-4.6</td>
</tr>
<tr>
<td>Contact someone to get help</td>
<td>49 (62.8)</td>
<td>51.1-73.5</td>
</tr>
</tbody>
</table>
4.4 Discussion

This study provides new information on potential risks of influenza A virus transmission on farms in rural Thailand, based on a questionnaire survey regarding farm management and pig-trading activities. The main aim of the risk assessment was to generate information that could be used to highlight the importance of developing specific public health policies for prevention of influenza virus spread on Thai small pig farms. As the study was conducted in only one community, the possible lack of representativeness for the overall small pig farm population in Thailand was a major limitation of this study. However, the small sample size was due to the fact that only a limited number of farms from a small area supply pigs to the niche tourist trade market. Nevertheless, this area is considered very important due to the potential cross-border trade in pigs between Thailand and Lao PDR and a low level of biosecurity undertaken by farmers. The study does represent pig farms in rural communities, where farmers raise pigs with limited resources for a secondary income, and their practices are generally influenced by socio-economic considerations.

The findings of this study are consistent with the direct observations on inter-species interactions in small swine farms in Peru, including mixed farming of swine with chickens, common human–pig contacts and suboptimal biosecurity and hygiene practices adopted by farmers (McCune et al. 2012). The situation observed in Mukdahan was similar to that reported from the neighbouring countries (Cambodia, Laos and Vietnam), where nearly 70% of all pigs are raised in small-scale farms (Huynh et al. 2007). In Cambodia for example, smallholders and mixed farming
practices (animals-crops) are considered the backbone of the agriculture system, with poor families commonly raising chickens and one or two pigs per household (Tornimbene and Drew 2012).

On most studied farms, the intermingling of species was commonly found and free-ranging chickens were observed. Although avian influenza viruses seem to be poorly transmissible to swine, a study by Suriya et al. (2008) found that mammalian pets on pig farms can serve as potential carriers to spread the viruses to pigs. Keeping pigs close to other species should therefore be discouraged to diminish the risk of cross-species transmission and the possibility of reassortment of influenza A viruses. Generally, pigs were raised intraditional pens that could facilitate the dissemination of airborne pathogens through close contact between animals from different pens. In addition, the high humidity and poor hygiene of the pens may favour the survival and transmission of influenza viruses in the environment (Kyriakis et al. 2011). The use of readily available products in households that are able to kill the A(H1N1)pdm09 virus, such as 1% bleach, 10% vinegar and 0.01% washing up liquid, should be encouraged for cleaning pens in this low-resource setting (Greatorex et al. 2010). Encouragingly, hand-washing with soap was performed by most farmers. If carried out properly, such a practice is effective in removing the A(H1N1)pdm09 virus from the hands (Grayson et al. 2009).

No limiting of visitors or other biosecurity protocols were applied in the studied farms. Uncontrolled access could be associated with a high seroprevalence to influenza viruses (Simon-Grife et al. 2011). Using manure as field fertilizer may play
a role in transmission of influenza viruses in the region (Desrosiers et al. 2004). The regular practice of hiring boars from outside could also be a significant contribution to disease transmission in the local community. To illustrate, the hired boars could carry viruses and boar owners may transmit the viruses back to their premises through their boots, clothing or vehicles. Limiting visits and improving biosecurity practices are key in preventing virus introduction and lowering the chance of virus spread between farms.

Most farmers generally spent one hour per day feeding pigs. The A(H1N1)pdm09 virus could become established in pig populations following primary contact with infected humans (Brookes and Brown, 2011). A reduction in this pig-to-human interaction may result in decreased transmission but may result in increased losses and reduced productivity through reduced observation of animals. Whilst working with pigs, most farmers only used rubber boots for protection and some wore no protective footwear. Without proper protective clothing there is a high likelihood of exposure to influenza viruses from infectious animal secretions (Kumarsean et al. 2009). Thus, personal protective measures should be adopted to reduce virus transmission between pigs and humans on farms. Furthermore, a policy of annual influenza vaccination of swine workers may lower the possibility of reassortment events occurring (Myers et al. 2007). Unfortunately, pandemic vaccine candidate recommendations in Thailand do not include pig farmers. During pandemics, agricultural workers should be included in influenza surveillance efforts and be considered a priority group for annual influenza vaccination and antivirals (Gray et al. 2007).
Although the potential spread of the virus through the movements of pig traders was mainly local, transmission of influenza A viruses in the community could easily occur from one farm to another by indirect contact through vehicles, humans and fomites. Of note, local pigs were slaughtered at an early age that may facilitate disease spread in the community through frequent trading; however, the risk of virus circulation in the pig population may conversely be reduced due to rapid replacement. In a surveillance context, trading seasonality should be taken into account. It was suggested that surveillance systems for pig diseases should be targeted around the festival months, which in Thailand are January and April.

On most farms, pig identification was lacking and this could compromise disease control and surveillance efforts. With animal identification, if an outbreak occurred, control and eradication processes could be implemented in a timely manner. On-farm identification should therefore be promoted to ensure traceability of animals. Overall, risk behaviours for influenza virus transmission from pigs to humans and vice versa that could arise from pig-farming practices and trading activities have been demonstrated in this study.

To improve statistical analysis, multivariate logistic regression with random effects could be performed in future in order to identify confounding factors and risk factors. An interdisciplinary approach involving epidemiologists, economists and sociologists should be carried out to refine the strategies to the actual context. Strengthening collaboration between the relevant authorities responsible for the human health, animal health and food safety sectors, that is, following the One
Health approach, will improve surveillance activities and control of inter-species transmission of influenza A viruses. To account for complete mechanisms of human infection with SIVs, more investigations of the key parameters, including pig–human contact rate, the disease prevalence in pigs and the probability of human infection, are needed in the future.

The next chapter studies the presence of influenza A viruses in pigs from farms where potential risks of virus transmission were examined. Due to limited resources, target sampling was performed to detect influenza A viruses in pigs from small farms in this rural community. Nasal swab samples, together with serum specimens, were collected for virus isolation and antibody detection, respectively.
Chapter 5: Surveys to Detect the Presence of Influenza A viruses in pigs in Rural Thailand

5.1 Introduction

Swine influenza is an acute respiratory disease of pigs caused by influenza A viruses, belonging to the family Orthomyxoviridae (Vincent et al. 2009). Influenza was first recognised as a pig disease during the 1918 pandemic (Easterday and Van Reeth 1999). Later, the influenza virus was isolated from pigs in the USA in 1930 (Shope 1931). Traditionally, influenza A viruses have been subtyped according to the antigenic properties of 2 surface glycoproteins, namely haemagglutinin and neuraminidase (Ma et al. 2009). Three main subtypes of influenza infections in pig populations worldwide are H1N1, H1N2 and H3N2 (Webby et al. 2004). Swine influenza is of public health concern because the viruses can be transmitted from pigs to people i.e. pigs can act as reservoirs of zoonotic influenza viruses for humans (Easterday and Van Reeth 1999).

The segmented nature of the influenza viral genome allows reassortment to occur in a host when it is simultaneously infected with 2 distinct subtypes of influenza A viruses (Webster et al. 1992). Pigs are an important host in influenza virus ecology, since they are susceptible to infection with influenza viruses of avian and mammalian lineages due to the presence of receptors for both lineages in the
respiratory tract (Ito et al. 1998). Thus, pigs can serve as “mixing vessels” for different lineages, providing an opportunity for the creation of novel reassortants (Olsen et al. 2004). Importantly, the reassortant viruses may acquire mammalian adaptation characteristics, thereby allowing human infection and the potential for human pandemics.

The potential role of pigs in the generation of pandemic influenza strains gained attention in the recent 2009 pandemic as the A(H1N1)pdm09 virus was closely related to a number of currently circulating pig viruses in the 'Classic North American' and 'Eurasian' swine influenza virus lineages (Smith et al. 2009). The maintenance of these viruses in pigs and the frequent exchange of viruses with other species are directly facilitated by regular close contact between pigs and humans or birds during husbandry practices (Brown 2000). South East Asia, in particular, is considered a potential epicenter for emergence of pandemic viruses given the close proximity with which humans live to their domestic animals (Nelson et al. 2007).

In Thailand, livestock production has shifted in the past 20-25 years from backyard farming and integrated cropping-livestock farming systems to industrial livestock farming enterprises (Riethmuller and Chalermpao 2002; Charoensook et al. 2013). Around 80% of pigs produced in Thailand are reared in intensive farming systems and 56% of these are from farms with over 1000 pigs. The remainder are from small (50 – 200 pigs) to medium (201 – 1000 pigs) farms. Large intensive farms are either integrated company owned (8.5%) or private independent (47.5%) enterprises (Cameron 2000). The primary area for swine production is the central region of the
country, where approximately 57% (4,669,535 head) of the country’s pig population (8,537,703 head) are located (Charoensook et al. 2013).

Previous studies of influenza A viruses in pigs in Thailand were mostly conducted in the provinces with high pig densities in the central area of the country, and were focused on slaughter houses, farms with clinically normal animals and farms where outbreaks had been reported (Kupradinun et al. 1991; Chutinimitkul et al. 2008; Nakharuthai et al. 2008; Takemae et al. 2008; Lekcharoensuk et al. 2010; Sreta et al. 2010; Kitikoon et al. 2011a; Takemae et al. 2011; Charoenvisal et al. 2013). However, investigations in remote areas have often been neglected due to logistical and economical constraints (Trevennec et al. 2011). Given the lack of laboratory data on influenza A viruses in pigs in rural Thailand, the current study was undertaken in order to improve the understanding of the influenza situation in pigs in Mukdahan province, where small pig farms with low biosecurity levels are common.

5.2 Materials and methods

5.2.1 Selection of pigs for sampling

All indigenous pigs on the 98 farms where risk factors had previously been investigated were sampled in this study (see Chapter 4 for sampling methodology and other details). These pigs have certain distinct characteristics, including black coloration and are preferred by customers in this region (Figure 5.1).
To increase the chances of isolating influenza A viruses, only samples from weaner pigs were targeted for virus detection (see Figure 5.2 for sampling details), as the maternal antibodies decline with age in piglets, making this group more likely to be susceptible to virus infection (Takemae et al. 2011). For detection of antibodies to influenza A viruses, only sows were targeted, since protective antibodies are usually found in reproductive age animals, as they are kept for longer periods and are more likely to have experienced influenza infections during their lives (Kyriakis et al. 2011). In addition, piglets were not included in the serological survey as maternal antibodies to influenza A viruses can persist for 2 to 4 months (Easterday and Van Reeth 1999) and thus can complicate the serological results and subsequent analyses.

**Figure 5.1.** Thai indigenous pig at a small farm in Mukdahan, Thailand.
Figure 5.2. Overview of sample selection for laboratory testing.

5.2.2 Sample collection

For nasal swab samples, a total of 211 specimens were collected from weaning pigs aged 1 to 3 months. Specimens from both nostrils were obtained with the same swab. The tip of the swab was put into a tube containing 2-3 ml of viral transport medium and the applicator stick was broken off. For serology, a total of 118 samples were collected from sows aged 6 months to 3 years (Figure 5.3). The sera were allowed to clot at room temperature and were then centrifuged at 2500 rpm for 15 minutes to separate the red blood cells and sera. The serum was removed by pipette and the red blood cells were discarded. No pigs were displaying clinical signs of respiratory
illness during sampling. Sera and swab samples were frozen at -20°C prior to being sent to the NIAH, Thailand for testing.

Figure 5.3. Blood collection from a sow in the study for antibody detection.

5.2.3 Virus isolation

For virus isolation, MDCK cells were used according to the method described by the Office International des Epizooties (OIE 2000). Briefly, swab tubes were vortexed vigorously for 3-5 minutes and cotton swabs were then removed from the tube with sterile forceps. The tube was centrifuged at 1000 g at 4°C for 10 minutes and the
swab supernatant was harvested. For virus isolation, an MDCK continuous cell line was used in 24 well plates. Prior to inoculation, the medium from the seeded wells was removed by gentle inversion and washed 2-3 times with phosphate-buffered saline (PBS) containing a final concentration of 1 µg/ml of TPCK-treated trypsin. For each sample, 0.1-0.2 mL of the swab preparation was inoculated onto MDCK cells. The inocula were allowed to absorb at 37°C for 60-120 minutes in a 5% CO₂ humidified incubator. The cell monolayer was washed 1-2 times with PBS containing a final concentration of 1 µg/ml of TPCK-treated trypsin. The inocula were added to the media for virus growth (Dulbecco’s Modified Eagle Medium) and incubated at 37°C in a 5% CO₂ atmosphere for 3 to 5 days with daily observation for CPE. After 3 to 5 days, the supernatant was removed with a pipette and were tested for haemagglutination activity. If the test was negative, the samples were inoculated onto MDCK cells and tested by the haemagglutination test after 5 days.

5.2.4 Serological assay

For detecting antibodies to any influenza A subtype, the serum samples were tested with a commercial ELISA kit, namely IDEXX Influenza A Ab Test (IDEXX, ME) with claimed applicability to avian, canine, feline and swine species. Regardless of influenza subtype or antigenic variance between species, the ELISA kit targets conserved anti-influenza A virus nucleoprotein antibodies. It can therefore detect exposure in any species that generates an antibody response to the nucleoprotein.

An ELISA was performed according to the protocol recommended by the manufacturer. Briefly, samples to be tested were diluted 1:10 in the diluent provided
in the kit (15 µL of sample into 135 µL of sample diluent). One hundred and fifty microlitres of each control or diluted sample were transferred to a well and incubated for 60 minutes at room temperature. After incubation, the controls and samples were discarded from the wells and the plates were washed three times with wash solution. One hundred microlitres of peroxidase-labeled anti-IgG secondary antibody, which was also provided in the kit, were dispensed to each well and incubated for another 30 minutes at room temperature. After rinsing, the presence of antigen–antibody complexes was observed by adding 3,3',5,5'-Tetramethylbenzidine (TMB) substrate to each well and incubating the plates for 15 minutes at room temperature. The results were expressed as S/N values (sample-to-negative ratios). Samples with an S/N lower than 0.6 were considered to be positive for antibody against influenza A viruses in pigs.

5.3 Laboratory results

Laboratory results indicated that influenza A viruses were not common in pigs in Mukdahan. From a total of 211 swab samples, no viruses were isolated (0.0%, 95%CI: 0.0-1.7%). In addition, all 118 sera were negative by ELISA (0.0%, 95%CI: 0.0-3.1%).

5.4 Discussion

No evidence for the presence of influenza A virus infections was found in pigs in the study site in rural Thailand. This could be due to the absence of several previously identified risk factors for influenza virus infection at the herd level. The most common risk factors include high pig and/or farm densities, large herd sizes, high replacement rates and import or purchase of pigs (Ewald et al. 1994; Maes et
In the area studied, pigs were local breeds, with small numbers being raised in low densities under traditional management systems. Despite the low level of biosecurity, pig farming with conventional open-air systems in rural Thailand may not be favourable for virus circulation. A similar situation was shown in a study in a remote area of Northern Vietnam, where freedom from swine influenza was demonstrated on farms (Trevennec et al. 2012). The results suggested that the low pig density, i.e. small pig farms with an average of 4–5 pigs, and scattered villages, limit the virus exposure from neighbouring farms and villages, and thus local spread through aerosols was unlikely to occur. Further epidemiological investigations should be carried out in order to elucidate the circulation of influenza A viruses in local pig farms in rural Thailand.

The negative findings in this study could also be related to pig breed. Certain genetic characteristics may impact the risk of swine influenza virus infection. To be specific, it has been reported that the porcine $Mx1$ gene is implicated in resistance to influenza viruses (Nakajima et al. 2007). Therefore, the variation in the $Mx1$ gene among pig breeds may result in host genetic differences in susceptibility to influenza infection. All positive results from the previous studies on influenza A viruses in pigs in Thailand have been conducted in commercial pig farms where imported breeds or cross-breeds between Large White, Landrace and Duroc were common. Additional studies are recommended to demonstrate whether indigenous pigs possess genetic resistance to influenza virus infections. This information may be useful in improving pig breeds with the genetic trait of resistance to influenza infection.
There are a number of causes for false negatives with virus isolation. For example, viruses can be inactivated during shipping or by disinfectants, some influenza viruses may not grow to detectable levels in cells, or the sample may not have been collected correctly or collected at the correct time of infection, or it may have been handled improperly after collection (Swenson et al. 2001). According to the laboratory procedures described by the World Health Organization (WHO 2012), clinical specimens for viral isolation should be placed in ice packs and transported to the laboratory promptly. If clinical specimens are transported to the laboratory more than 2 days, they should be frozen at or below -70°C until transported to the laboratory. However, in the current study, virus isolation was not performed in the local laboratories and the swab specimens were stored at -20°C due to the lack of facilities. Thus, poor sample management may have resulted in an underestimation of the true proportion of pigs with virus infection in this study.

For isolation of the influenza A viruses in pigs from clinical specimens, a combination of embryonated eggs and cell culture is commonly used (Hiromoto et al. 2012). Recently, it was found that the A(H1N1)pdm09 viruses isolated from pigs grew poorly in MDCK cells (Hiromoto et al. 2012). Given limited resources, virus isolation from field samples in the present study was carried out using MDCK cells alone. The negative results could thus be related to poor virus growth in MDCK cells. Regarding the detection of antibodies against influenza A viruses in pigs, the HI test is the principal serological test used and is subtype and strain specific (OIE 2008). It has been used most commonly in veterinary diagnostic laboratories to detect anti-influenza virus antibody and is considered to be the standard test for international trade of animals by OIE (2000). Based on the purpose of the current
study, an ELISA was carried out alone to screen for influenza A viruses without
further verification by HI testing. However, if sera show positive results in the
ELISA kits, they should then be tested further in HI tests with representative virus
antigens to identify the virus lineages that are likely to be prevalent in the local
geographic region (Tse et al. 2012).

The IDEXX Influenza A Ab test was reported to have a 99.6% specificity (95% CI,
97.7%–100%) and 95.3% sensitivity (95% CI, 91.8%–97.3%) for detecting exposure
to influenza A in pigs, which is comparable to the HI. However, lower performance
levels for the IDEXX Influenza A Ab test have been demonstrated by Tse et al.
(2012), who found 79% specificity (95%CI: 63–90%) and 86% sensitivity (95%CI:
76-93%). Yoon et al. (2004) stated that a commercial ELISA kit for detecting
antibody against H1N1 SIV might not identify positive animals as effectively as the
HI test, since it could miss animals at the early stage of infection. Thus, the
validation of laboratory procedures and the evaluation of test performance
characteristics for influenza A viruses in pigs should be carried out in future studies.

For control and prevention measures, the implementation of appropriate infection
control measures was a key aspect for its control. The next chapter studies the KAPs
of smallholder pig farmers regarding influenza A viruses to help develop appropriate
strategies for further intensive campaigns for influenza prevention in the community.
Fifty pig farmers were administered questionnaires through face-to-face interviews
and demographic data on age, sex, marital status and education level of pig farmers.
were collected, as well as their KAPs regarding influenza A infection in pigs, with particular reference to the A(H1N1)pdm09 virus.
Chapter 6: Determining knowledge, attitudes and practices of Thai pig smallholders towards influenza

6.1 Introduction

Influenza A viruses are major human and animal pathogens, which not only cause epidemics of respiratory disease in populations worldwide but also have public health implications due to the possibility of reassortment. Importantly, pigs are susceptible to influenza viruses of human, avian and swine origins and thus are considered as a potential host for development of novel viruses, some of which may be transmitted to other species including humans, potentially resulting in catastrophic pandemics in humans (Ito et al. 1998; Ma et al. 2009).

In 2009, there was no evidence of the A(H1N1)pdm09 virus in pigs before it was detected in humans (Brookes and Brown 2011). However, this strain is presumed to have originated from pigs as it contains genes from two different lineages of contemporary SIVs (Smith et al. 2009). Shortly after the appearance of the A(H1N1)pdm09 virus in humans, the virus was transmitted from people back into pigs (Nelson et al. 2012). Subsequently reassortant viruses between this virus and endemic SIV were reported across the world (Nelson et al. 2012), suggesting that the virus had become established in pig populations. Importantly, the reassortant H3N2
SIV with matrix segments of the A(H1N1)pdm09 virus caused respiratory illness in humans in the USA (CDC 2012).

Pig farmers are an occupational group at risk of acquiring influenza A viruses from pigs during the course of their work. Several studies have observed that people with occupational exposure to pigs had increased rates of SIV infections (Myers et al. 2007). However, zoonotic infections with SIV in people involved in pig production may be more frequent than reported in the literature because human cases of SIV infections may not be clinically distinguishable from infections caused by human influenza viruses (Olsen et al. 2002). Of particular concern is the small-scale or family pig production sector where disease awareness and biosecurity may be lacking (Trevennec et al. 2011). Therefore, the study reported in this Chapter was designed to assess the KAPs of smallholder pig farmers in rural Thailand regarding influenza A viruses. The results will help to inform public health officials and provide information to help in the development of appropriate community-based strategies to prevent influenza illness and control programmes in the future.

6.2 Materials and methods

6.2.1 Study population

A community cluster survey of KAPs among farmers regarding influenza was performed in small pig farms in Wanyai district, Mukdahan province. This district was considered a potentially high-risk area for the interspecies transmission of influenza A viruses (results in Chapter 4). Fifty pig farmers were randomly recruited
by the district veterinary officer and were administered questionnaires through face-to-face interviews. All participants provided informed consent.

### 6.2.2 Questionnaire

A standardized, structured questionnaire (Appendix 3) was developed to collect basic demographic data on age, sex, marital status and education level of pig farmers, as well as to assess their knowledge, attitudes and practices regarding influenza A infection in pigs, with particular reference to the A(H1N1)pdm09 virus. Questions on knowledge were used to determine the participants’ general knowledge regarding disease definition, clinical signs and modes of transmission. Questions on attitudes were developed to assess risk perceptions and control measures. Questions on practices were used to evaluate personal hygiene and disease prevention behaviours. A summary of the questions included in the questionnaire is shown in Table 6.1. The questionnaire was approved by the Murdoch University Human Ethics Committee (Project no. 2011/172).

The open-ended questions on basic knowledge were examined and categorised into “correct”, “incorrect” and “do not know” options. If farmers were uncertain in defining the disease, choices were provided for responses (acute or chronic; infectious or non-infectious; caused by influenza A viruses or not). For the question on mode of virus transmission the response choices were coded on a three-point Likert-type scale using “yes”, “no” and “do not know” options. The responses for all statements in relation to risk perception towards influenza A viruses in pigs were categorised on a five-point Likert-type scale (from 1 to 5, 1=Unable to Judge, 2=No
risk, 3=Low risk, 4=Medium risk, 5=High risk). In nine questions, respondents were asked to use a seven-point Likert-type scale to rate their agreement with control measures for influenza A viruses in pigs, with responses ranging from 1 (strongly disagree) to 7 (strongly agree). Farmers were also asked to rate their risk perception of contracting influenza A(H1N1)pdm09 from 1 (no concern) to 6 (very high concern). For questions of disease awareness and prevention practices participants were allowed to choose from a pre-existing set of answers. Five response choices for the questions regarding their personal hygiene practices ranged from all the time to never (all the time, most of the time, some of the time, rarely and never) and one option for not sure. The variables derived from these questions were mostly categorical, with the exception of age.
Table 6.1. Summary of questions for knowledge, attitudes and practices towards influenza.

<table>
<thead>
<tr>
<th>Knowledge</th>
<th></th>
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<tbody>
<tr>
<td><strong>Basic Knowledge</strong></td>
<td></td>
</tr>
<tr>
<td>What is influenza A in pigs?</td>
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<tr>
<td>What signs would you associate with influenza A in pigs?</td>
<td></td>
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<tr>
<td>What are the main modes of transmission of influenza A viruses in pigs</td>
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<tr>
<td><strong>Attitudes</strong></td>
<td></td>
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<tr>
<td>Disease risk perception on influenza A in pigs</td>
<td></td>
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<tr>
<td>What factors do you think pose a risk of your pigs contracting influenza A viruses?</td>
<td></td>
</tr>
<tr>
<td>(1) Introduction of new pigs</td>
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<tr>
<td>(2) Neighbours’ pigs close to your pigs</td>
<td></td>
</tr>
<tr>
<td>(3) People, equipment and vehicles entering farms</td>
<td></td>
</tr>
<tr>
<td>(4) Many kinds of animals raised on farm</td>
<td></td>
</tr>
<tr>
<td>(5) Pigs with flu-like symptoms</td>
<td></td>
</tr>
<tr>
<td>(6) People with flu-like symptoms</td>
<td></td>
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<tr>
<td>Control measures for influenza A viruses in pigs</td>
<td></td>
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<tr>
<td>What do you see as necessary to prevent or control influenza A viruses in pigs?</td>
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<tr>
<td>(1) Early disease detection of influenza A viruses in pigs</td>
<td></td>
</tr>
<tr>
<td>(2) More education and awareness on prevention of influenza A viruses in pigs</td>
<td></td>
</tr>
<tr>
<td>(3) Authorities to advise me when pigs are sick with respiratory signs</td>
<td></td>
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<tr>
<td>(4) Regular veterinary visits</td>
<td></td>
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<tr>
<td>(5) Safe source of pigs</td>
<td></td>
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<tr>
<td>(6) Control pig movement from outbreak areas of influenza A viruses in pigs</td>
<td></td>
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<tr>
<td>(7) Prevention of contact between pigs on farm and neighbouring farms</td>
<td></td>
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<tr>
<td>(8) Vaccination against swine influenza in pigs</td>
<td></td>
</tr>
<tr>
<td>(9) Vaccination against seasonal influenza in humans</td>
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<tr>
<td><strong>Awareness on influenza A in pigs</strong></td>
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<tr>
<td>Where did you learn about influenza A viruses in pigs?</td>
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<tr>
<td>Are you interested in receiving further information on influenza A viruses in pigs?</td>
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<tr>
<td>What special information on influenza A viruses in pigs would you like to know?</td>
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<tr>
<td>What are the best ways to get this information to you?</td>
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<tr>
<td><strong>Risk perception on influenza A(H1N1)pdm09 in humans</strong></td>
<td></td>
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<tr>
<td>Where did you learn about influenza A(H1N1)pdm09?</td>
<td></td>
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<tr>
<td>Do you have concerns that you might get sick from A(H1N1)pdm09 virus?</td>
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<tr>
<td>Have you taken special precautions to protect yourself against A(H1N1)pdm09 virus?</td>
<td></td>
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<tr>
<td>Have you ever had human influenza vaccination? If not, why not?</td>
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<tr>
<td><strong>Practices</strong></td>
<td></td>
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<tr>
<td>Prevention practice</td>
<td></td>
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<tr>
<td>What hygienic practices do you adopt to avoid spreading of influenza A viruses in pigs?</td>
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<tr>
<td>How do you ensure only healthy pigs are purchased?</td>
<td></td>
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<tr>
<td>Please explain what you would do if you suspected your pigs had influenza A viruses?</td>
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<tr>
<td>Personal practice</td>
<td></td>
</tr>
<tr>
<td>How do you usually wash your hands?</td>
<td></td>
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<tr>
<td>Do you wash your hands after touching pigs?</td>
<td></td>
</tr>
<tr>
<td>Do you touch sick or dead pigs with your bare hands?</td>
<td></td>
</tr>
<tr>
<td>Will you work if you have flu-like symptoms?</td>
<td></td>
</tr>
<tr>
<td>Do you avoid contact with people who have symptoms of flu?</td>
<td></td>
</tr>
</tbody>
</table>
6.2.3 Statistical analysis

Data were entered into Microsoft Excel and analysed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Mean and standard deviation (SD) values were calculated for continuous variables. For categorical variables, percentage and 95% confidence intervals were calculated based on the exact binominal method (Ross 2003).

6.3 Results

6.3.1 Demographics

A total of 50 farmers participated in the study. Socio-demographic characteristics of the study population are summarised in Table 6.2. The average age±SD of respondents was 48.6±11.3 years. Most farmers were female (84%, 95%CI: 70.9-92.8), all were married (100%, 95%CI: 92.9-100.0) and the majority had completed only primary school level education (60%, 95%CI: 45.2-73.6).

Table 6.2. Socio-demographic characteristics of the study population (n=50).

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>%</th>
<th>95%CI</th>
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</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>16</td>
<td>7.2-29.1</td>
</tr>
<tr>
<td>Female</td>
<td>42</td>
<td>84</td>
<td>70.9-92.8</td>
</tr>
<tr>
<td>Marital</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Married</td>
<td>50</td>
<td>100</td>
<td>92.9-100.0</td>
</tr>
<tr>
<td>Single</td>
<td>0</td>
<td>0</td>
<td>0.0-7.1</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No formal</td>
<td>0</td>
<td>0</td>
<td>0.0-7.1</td>
</tr>
<tr>
<td>Primary school</td>
<td>30</td>
<td>60</td>
<td>45.2-73.6</td>
</tr>
<tr>
<td>Secondary</td>
<td>7</td>
<td>14</td>
<td>5.8-26.7</td>
</tr>
<tr>
<td>High school</td>
<td>9</td>
<td>18</td>
<td>8.6-31.4</td>
</tr>
<tr>
<td>Technical</td>
<td>3</td>
<td>6</td>
<td>1.3-16.5</td>
</tr>
<tr>
<td>University</td>
<td>1</td>
<td>2</td>
<td>0.1-10.6</td>
</tr>
<tr>
<td>Age (year)</td>
<td>48.6±11.3*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± Standard deviation
6.3.2 Knowledge

The farmers' knowledge on influenza A in pigs is summarised in Table 6.3. Only 28% of farmers correctly defined influenza A in pigs as an acute infectious disease caused by influenza A viruses. Sixty-five per cent correctly recalled the clinical signs of influenza A in pigs. Most farmers considered pig-to-pig transmission as a potential mode of virus transmission (64%, 95%CI: 49.2-77.1). Half of the respondents knew that the virus could be transmitted from pigs to humans (95%CI: 35.5-64.5); however, more than half did not know of human-to-pig transmission of influenza A viruses (52%, 95%CI: 37.4-66.3).

Table 6.3. Knowledge of pig farmers (n=50) about influenza A in pigs.

<table>
<thead>
<tr>
<th></th>
<th>Correct</th>
<th>Incorrect</th>
<th>Do not know</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n %</td>
<td>95%CI</td>
<td>n %</td>
</tr>
<tr>
<td><strong>Definition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute infectious disease</td>
<td>14 28.0</td>
<td>16.2-42.5</td>
<td>12 24.0</td>
</tr>
<tr>
<td>caused by influenza A viruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Clinical signs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nasal discharge, sneezing, coughing, high fever</td>
<td>32 65.3</td>
<td>50.4-78.3</td>
<td>2 4.1</td>
</tr>
<tr>
<td><strong>Modes of transmission</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig-to-pig</td>
<td>32 64</td>
<td>49.2-77.1</td>
<td>2 4.0</td>
</tr>
<tr>
<td>Pig-to-human</td>
<td>25 50</td>
<td>35.5-64.5</td>
<td>3 6.0</td>
</tr>
<tr>
<td>Human-to-pig</td>
<td>12 24</td>
<td>13.1-38.2</td>
<td>12 24</td>
</tr>
<tr>
<td>Eating uncooked pork</td>
<td>29 58</td>
<td>43.2-71.8</td>
<td>1 2.0</td>
</tr>
<tr>
<td>Touching uncooked pork</td>
<td>15 30</td>
<td>17.9-44.6</td>
<td>13 26</td>
</tr>
<tr>
<td>Eating cooked pork</td>
<td>1 2.0</td>
<td>0.1-10.6</td>
<td>30 60</td>
</tr>
</tbody>
</table>
6.3.3 Attitudes

The risk perception of influenza A in pigs among farmers is shown in Table 6.4. The majority of farmers perceived pigs with flu-like symptoms as high risk, contributing to virus transmission (62%, 95%CI: 47.2-75.3). On the other hand, a high proportion of farmers believed that people with flu-like symptoms posed no risk of infecting pigs (30%, 95%CI: 17.9-44.6). Introduction of new pigs, raising many kinds of animals on farms, neighbours’ pigs and people, equipment and vehicles entering farms were considered as medium risk or high risk by most farmers.

Table 6.4. Attitudes towards risk perception of pigs contracting influenza A viruses

(1=Unable to Judge, 2=No risk, 3=Low risk, 4=Medium risk, 5=High risk).

<table>
<thead>
<tr>
<th>Risk perception</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction of new pigs</td>
<td>6</td>
<td>12</td>
<td>4.5-24.3</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Neighbours’ pigs</td>
<td>6</td>
<td>12</td>
<td>4.5-24.3</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>People, equipment and vehicles</td>
<td>4</td>
<td>8</td>
<td>2.2-19.2</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>Many kinds of animals</td>
<td>3</td>
<td>6</td>
<td>1.3-6.5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Pigs with flu-like symptoms</td>
<td>3</td>
<td>6</td>
<td>1.3-6.5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>People with flu-like symptoms</td>
<td>9</td>
<td>18</td>
<td>8.6-31.4</td>
<td>15</td>
<td>30</td>
</tr>
</tbody>
</table>
The perception of pig farmers towards control measures for influenza A viruses in pigs was studied (Figure 6.1). It was found that most farmers strongly agreed that early disease detection (69.4%, 95%CI: 54.6-81.7%), more education and awareness (69.4%, 95%CI: 54.6-81.7%), the advice of the authorities (73.5%, 95%CI: 58.9-85.1%), veterinary visits (75.5%, 95%CI: 61.1-86.7%), safe sources of pigs (59.2%, 95%CI: 44.2-73.0%), movement control (79.6%, 95%CI: 65.7-89.8%), prevention of pig contact between farms (65.3%, 95%CI: 50.4-78.3%) and pig vaccination against SIV (63.3%, 95%CI: 48.3-76.6%) were necessary for controlling influenza A viruses in pigs. Notwithstanding these findings, a high percentage of farmers disagreed that human vaccination against seasonal influenza was necessary for disease control and prevention (26.5%, 95%CI: 14.9-41.1%).

Figure 6.1. Farmers’ perception towards control measures for influenza A viruses in pigs (N=50).
Almost all farmers reported interest in receiving further information about influenza A viruses in pigs (96.0%, 95%CI: 86.3-99.5%) and the topics they were most interested in were basic knowledge and prevention measures (50.0%: 95%CI: 35.2-64.8%). Seventy-one per cent of farmers believed that the best way to obtain information on influenza A viruses in pigs was through paravets (95%CI: 55.9-83.0%). Regarding influenza A(H1N1)pdm09 in humans, 94% of farmers had heard of influenza A(H1N1)pdm09 (95%CI: 83.5-98.7%). The majority of them acquired information through media sources, such as TV and radio (68.1%, 95%CI: 52.9-80.9%). Forty-two percent of farmers had a very high concern about contracting influenza A(H1N1)pdm09 (95%CI: 28.3-57.8%), whereas only one farmer had no concern (2.1%, 95%CI: 0.1-11.3%). Due to disease awareness, most farmers reported changes in their behaviour, including increased hand washing as well as purchasing and wearing of face masks (68.1%, 95%CI: 52.9-80.9% and 59.6%, 95%CI: 44.3-73.6%, respectively). However, more than half had never been vaccinated against human influenza (61.2%, 95%CI: 46.2-74.8%).

6.3.4 Practices

Preventive measures adopted by interviewed farmers are summarised in Table 6.5. The hygienic practice most commonly adopted by farmers to avoid spreading of influenza A viruses between pigs was hand washing after handling pigs or manure (94.0%, 95%CI: 83.5-98.7%), the second most common practice was changing clothes after touching pigs or their faeces (78.0%, 95%CI: 64.0-88.5%) followed by wearing gloves when touching pigs (46.0%, 95%CI: 31.8-60.7%). A quarantine period was not applied by any farmers before introducing new pigs onto farms.
(95% CI: 0.0-7.1). To ensure that healthy pigs were purchased, most respondents only observed their physical condition (86%, 95% CI: 73.3-94.2%). The majority of farmers responded that they would report suspected cases to the local veterinary authorities if they found a sick pig (92%, 95% CI: 80.8-97.8%).

Table 6.5. Preventive measures adopted by farmers (N=50).

<table>
<thead>
<tr>
<th>What hygienic practices do you adopt to avoid spreading of influenza A viruses in pigs?</th>
<th>Yes</th>
<th>%</th>
<th>95% CI</th>
<th>No</th>
<th>%</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2</td>
<td>4</td>
<td>0.5-13.7</td>
<td>48</td>
<td>96</td>
<td>86.3-99.5</td>
</tr>
<tr>
<td>Only touch pigs if I am wearing gloves</td>
<td>23</td>
<td>46</td>
<td>31.8-60.7</td>
<td>27</td>
<td>54</td>
<td>39.3-68.2</td>
</tr>
<tr>
<td>Change clothes after touching pigs or their faeces</td>
<td>39</td>
<td>78</td>
<td>64.0-88.5</td>
<td>11</td>
<td>22</td>
<td>11.5-36.0</td>
</tr>
<tr>
<td>Wash hands with soap after handling pigs or manure</td>
<td>47</td>
<td>94</td>
<td>83.5-98.7</td>
<td>3</td>
<td>6</td>
<td>1.3-16.5</td>
</tr>
<tr>
<td>Wear face masks while working in farm</td>
<td>10</td>
<td>20</td>
<td>10.0-33.7</td>
<td>40</td>
<td>80</td>
<td>66.3-90.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>How do you ensure only healthy pigs are purchased?</th>
<th>Yes</th>
<th>%</th>
<th>95% CI</th>
<th>No</th>
<th>%</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsure of what to do</td>
<td>5</td>
<td>10</td>
<td>3.3-21.8</td>
<td>45</td>
<td>90</td>
<td>78.2-96.7</td>
</tr>
<tr>
<td>Only concerned with the price</td>
<td>0</td>
<td>0</td>
<td>0.0-7.1</td>
<td>50</td>
<td>100</td>
<td>92.9-100.0</td>
</tr>
<tr>
<td>Observe physical condition</td>
<td>43</td>
<td>86</td>
<td>73.3-94.2</td>
<td>7</td>
<td>14</td>
<td>5.8-26.7</td>
</tr>
<tr>
<td>I know the sellers and trust them</td>
<td>35</td>
<td>70</td>
<td>55.4-82.1</td>
<td>15</td>
<td>30</td>
<td>17.9-44.6</td>
</tr>
<tr>
<td>Quarantine before introducing new pigs into a herd</td>
<td>0</td>
<td>0</td>
<td>0.0-7.1</td>
<td>50</td>
<td>100</td>
<td>92.9-100.0</td>
</tr>
</tbody>
</table>

Table 6.5. Preventive measures adopted by farmers (N=50).

<table>
<thead>
<tr>
<th>Please explain what you would do if you suspected your pigs had influenza A viruses?</th>
<th>Yes</th>
<th>%</th>
<th>95% CI</th>
<th>No</th>
<th>%</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treat myself</td>
<td>3</td>
<td>6</td>
<td>1.3-16.5</td>
<td>47</td>
<td>94</td>
<td>83.5-98.7</td>
</tr>
<tr>
<td>Eat sick pigs</td>
<td>0</td>
<td>0</td>
<td>0.0-7.1</td>
<td>50</td>
<td>100</td>
<td>92.9-100.0</td>
</tr>
<tr>
<td>Give away to friends</td>
<td>0</td>
<td>0</td>
<td>0.0-7.1</td>
<td>50</td>
<td>100</td>
<td>92.9-100.0</td>
</tr>
<tr>
<td>Sell pigs to neighbours</td>
<td>0</td>
<td>0</td>
<td>0.0-7.1</td>
<td>50</td>
<td>100</td>
<td>92.9-100.0</td>
</tr>
<tr>
<td>Report suspect cases to an authority</td>
<td>46</td>
<td>92</td>
<td>80.8-97.8</td>
<td>4</td>
<td>8</td>
<td>2.2-19.2</td>
</tr>
<tr>
<td>Do nothing</td>
<td>0</td>
<td>0</td>
<td>0.0-7.1</td>
<td>50</td>
<td>100</td>
<td>92.9-100.0</td>
</tr>
</tbody>
</table>

111
Personal hygienic practices adopted by farmers are illustrated in Figure 6.2. The most common hygienic practice that farmers performed all the time was washing hands after touching pigs (82.0%, 95% CI: 68.6-91.4%) and the majority washed their hands with water and soap (94%, 95% CI: 83.5-98.7%). If farmers had flu-like symptoms, over one third of them would continue working (36.0%, 95% CI: 22.9-50.8%). Moreover, 38% of farmers did not avoid contact with people who had symptoms of flu (95% CI: 24.7-52.8%).

![Chart showing personal hygiene practices](image)

**Figure 6.2.** Personal hygienic practices adopted by farmers (N=50).
6.4 Discussion

In this study, a cross-sectional KAPs survey of pig smallholders in rural Thailand towards influenza A was undertaken in order to identify potential risk factors for influenza A and to help develop appropriate strategies for prevention of this disease in the community. This study found that most farmers had limited knowledge of influenza A in pigs. A high number of respondents could not define the disease and did not know the clinical signs and modes of transmission of influenza A virus in pigs. It is important to note that genetic characterisation of SIV isolates in Thailand has provided evidence of human-to-pig transmission of influenza A viruses in the country (Takemae et al. 2008; Lekcharoensuk et al. 2010). However, the majority of farmers perceived that people with flu-like symptoms posed a low risk to their pigs. Moreover, they did not consider human vaccination against influenza as an effective control measure for the disease. Therefore, it is crucial to provide farmers with the correct knowledge. Without sufficient knowledge, a positive attitude on its own may not change the behaviours of individuals into appropriate practices (Yap et al. 2010).

A willingness of farmers to embrace public health education campaigns about influenza A viruses in pigs was demonstrated as the respondents’ interest in learning more about the disease in pigs was very high. Specifically, basic knowledge and prevention strategies were the topics of most interest to farmers. It was found that the media was the main source of information on influenza A(H1N1)pdm09 for the study population, suggesting that the media was able to effectively reach people in rural Thailand. However, despite widespread information, most farmers had only limited knowledge of influenza A in pigs. This is in accordance with previous
studies on avian influenza in Thailand that showed that the information provided by
TV programs was superficial (Olsen et al. 2005; Maton et al. 2007). Therefore,
additional intervention is needed to enhance knowledge among rural farmers and this
could include public health campaigns based on economic considerations for
practical practices. Furthermore, the content of public health campaigns should be
designed in a format suitable for the poor and illiterate to ensure that educational
messages are understood and accepted by rural people (Leslie et al. 2008).

To successfully change individuals’ behaviours, concerted efforts are required (Ly et
al. 2007). For example in the context of routine medical care, it has been suggested
that medical professionals play a significant role in providing education, as people
who received information from health professionals or scientific journals had higher
compliance than those who did not receive information from these sources (Di
Giuseppe et al. 2008). In rural areas, the opinions of local leaders and doctors are
very important (Xiang et al. 2010), since they are believed to be trustworthy and
effective in providing information to the community. In a community-based
education trial implemented in Cambodia, a cascade training approach was effective
in providing education on biosecurity practices to the village community by training
local people to be trainers (Conan et al. 2013). In the present study, paravets were the
preferred group identified by farmers for disseminating educational messages and
thus they should be actively involved in future public health campaigns.

Vaccination against human influenza was not identified by farmers as important in
preventing disease transmission to pigs. In Thailand, vaccination of humans against
influenza is not widely adopted and influenza vaccine is usually restricted to persons who can afford to pay private health care providers for the immunization (Simmerman et al. 2004). The high cost of the influenza vaccine is probably the main obstacle to adoption of vaccination in Thailand (Simmerman et al. 2004). Given frequent on farm human-to-pig contact and the continued circulation of the A(H1N1)pdm09 virus in the human population, vaccination of pig farmers against influenza should be supported and encouraged to reduce the risk of disease transmission to pigs and the potential for further reassortment events (Terebuh et al. 2010).

A mathematical model employed to examine the transmission dynamics of a new influenza virus among populations demonstrated that if 50% of animal workers were vaccinated against the pandemic virus, the risk of viral transmission to the workers’ communities would be diminished (Saenz et al. 2006). In the 2009 pandemic, pig workers were not included in vaccination policies in Thailand. The groups recommended for vaccination included healthcare workers, pregnant women and persons with high-risk medical conditions. This was later expanded to include children 6 months to 2 years of age (Bunthi et al. 2013). It is worth noting that these vaccination policies could lead to misconception, as most respondents in the study personally believed that influenza vaccination was not necessary for them but only for persons with high-risk medical conditions.

Different levels of risk perception towards influenza A(H1N1)pdm09 have been found in several studies (Rubin et al. 2009; Seale et al. 2009; Balkhy et al. 2010).
Low levels of anxiety towards influenza A(H1N1)pdm09 were observed in studies from Australia and the United Kingdom (Rubin et al. 2009; Seale et al. 2009). In contrast, Balkhy et al. (2010) reported that more than half of the participants in Saudi Arabia had high concern. The present study showed that a large number of farmers had high levels of concern about contracting influenza A(H1N1)pdm09. It could be explained by the fact that the study was carried out during the post-pandemic period after Thailand had experienced rapid spread of the pandemic influenza A(H1N1)pdm09 virus. To illustrate, A(H1N1)pdm09 virus transmission was detected in all 76 Thai provinces and 65 deaths associated with virus infection were confirmed within 2 months after the first case was detected (Ungchusak et al. 2012). The high level of risk perception may thus be associated with the information that respondents had been exposed to before the study.

It has been shown previously that higher levels of concern may be related to an increase in behavioural changes (Lau et al. 2003). In this study, the majority of respondents changed their behaviours, such as increased hand washing and wearing face masks, to reduce their risk of contracting influenza. This was consistent with the non-pharmaceutical interventions emphasised in the national policy and prevention guidelines for influenza A(H1N1)pdm09 in Thailand, including the use of good hand hygiene practices, social distancing measures and the use of appropriate face masks (Ungchusak et al. 2012). The findings by Aiello et al. (2010) suggest that face masks and hand hygiene may reduce respiratory illnesses in shared living settings and lessen the impact of the influenza A(H1N1)pdm09 pandemic. In the current study, most farmers washed their hands after touching pigs and the majority used soap when washing their hands. Simple hand washing with soap and water has been
reported to be effective in preventing the transmission of influenza (Grayson et al. 2009). Furthermore, the use of an alcohol-based hand sanitizer is effective in inactivating a wide range of respiratory viruses and non-spore forming bacteria (Kampf and Kramer 2004; Aledort et al. 2007). Therefore, this intervention should also be encouraged.

One possible limitation of this study is the small sample size. Thus, the results of the study only reflect the KAPs of smallholder pig farmers in a rural community in North-Eastern Thailand. Furthermore, this study was a cross-sectional survey and may not have been able to correctly present the individuals’ real-world responses. In addition, farmers may change their responses over a period of time. Future longitudinal studies should be considered to validate these findings. In addition, the findings should be statistically analysed in a multivariate model to determine significant predictors of KAPs in farmers, for example age, education, income and other factors, as the results can be used to develop appropriate intervention strategies. Despite limitations, the study provided a good understanding of the KAPs towards influenza A among smallholder pig farmers in rural Thailand and the findings could be extrapolated to other communities where people have daily contact with pigs. This has significant public health implications and will be helpful when government veterinarians are developing on-farm influenza prevention and control programs in the future.

In the next chapter, sera obtained from pigs in Cambodia, where most pigs are raised by smallholders, were tested using HI assays for antibodies to human influenza A
viruses, together with HI and microneutralisation (MN) tests to assess the immunological responses to H5N1 virus.
Chapter 7: Serologic evidence of human influenza virus infections in pig populations in Cambodia

7.1 Introduction

Pigs are considered important intermediate hosts and possible ‘mixing vessels’ for genetic reassortment of influenza viruses owing to dual susceptibility to both human and animal influenza viruses (Castrucci et al. 1993; Scholtissek et al. 2002; Ma et al. 2009). Consequently, pigs have frequently been implicated in the emergence of human virus strains as was seen in the recent influenza pandemic where the A(H1N1)pdm09 virus contained a unique genome constellation derived from SIVs, namely the classical swine H1N1 lineage, the North American H3N2 triple-reassortant and the Eurasian ‘avian-like’ swine H1N1 virus (Garten et al. 2009; Peiris et al. 2009; Smith et al. 2009). The molecular characterisation of the A(H1N1)pdm09 strain revealed indirect evidence that pigs play a role in the ecology and emergence of influenza viruses (Zhu et al. 2011). However, there has been no direct evidence that pigs were involved in the epidemiology or spread of pandemic influenza virus in humans (Song et al. 2010).

The first case of A(H1N1)pdm09 virus infection in pigs was detected in a Canadian pig farm soon after the virus emerged in humans in April 2009 (Song et al. 2010). Thereafter, over 20 countries from five continents formally reported cases of A(H1N1)pdm09 in pigs to the OIE (Pasma and Joseph 2010; Pereda et al. 2010;
Brookes and Brown 2011). The increase in reported cases of A(H1N1)pdm09 in pigs, together with experimental studies by several teams (Brookes et al. 2009; Lange et al. 2009; Brookes and Brown 2011), has confirmed that A(H1N1)pdm09 virus can become established in pig populations. Furthermore, repeated detections of genetic reassortment between A(H1N1)pdm09-like viruses and other swine viruses in the USA, Europe and Asia suggest that a second generation of reassorted A(H1N1)pdm09 viruses might have been maintained in pigs for a period of time, and a process of adaptation of the A(H1N1)pdm09 virus to pigs might be occurring (Lange et al. 2009; Vijaykrishna et al. 2010; Ducatez et al. 2011; Howard et al. 2011; Kitikoon et al. 2011a; Moreno et al. 2011; Starick et al. 2011; Zhu et al. 2011). In the USA, the reassorted H3N2 SIVs with A(H1N1)pdm09 viruses have been detected among humans (CDC 2011b). Monitoring both human-to-pig and pig-to-human transmissions of influenza A viruses is therefore, critical to improve our understanding of these events and to minimise the likelihood of them occurring.

In Cambodia, nearly 70% of all pigs are raised in small-scale farms (Huynh et al. 2007). Pigs are bred traditionally, cohabit with humans under free-range conditions, and are usually raised to be sold for meat after relatively short periods (10–12 months) (Samkol 2008). Only a few commercial piggeries exist, which are mainly located near Phnom Penh City to supply the high urban demand for pork and other pig products (Chetra and Bourn 2009). The domestic pig producers cannot satisfy the demand for pork in the country (Dietze et al. 2011). It is estimated that over 1000 head of pigs or pig carcasses are imported each year from neighboring countries such as Thailand and Vietnam.
Many different influenza subtypes (including H4, H5, H6, H7, H9, H11 and H12) have been isolated from poultry and pigs in Asian countries, where the pig densities are highest worldwide (Palese 2004). The scientific communities and the international organizations like WHO, OIE and FAO agree that influenza surveillance activities around the world are urgently needed, especially in Southeast Asia where only a few countries provide data on influenza in swine (Smith et al. 2009; Van Reeth and Nicoll 2009; OFFLU 2011; Trevennec et al. 2011). In Cambodia, integrated production systems, consisting of one or more animal species with crops and fish, are predominant (Ramsay et al. 1999), facilitating transmission of influenza viruses from humans-to-swine, swine-to-human, or between pigs and avian species. Influenza viruses are, therefore, suspected to circulate actively, and the generation and dissemination of new variants are a real possibility in Cambodia.

A preliminary study for the detection of influenza A viruses in pigs was carried out by collecting nasal swab samples on a weekly basis from slaughtered pigs in Phnom Penh between 2006 and 2008 (Institut Pasteur in Cambodia unpublished data). However, among 1000 samples, no influenza viruses were isolated. In addition, no influenza-like symptoms were recorded suggesting that farmers preferred not to send sick animals to abattoirs, possibly to avoid investigations from animal health services. Owing to the previous study results, as well as concerns about the potential serologic cross-reactivity between A(H1N1)pdm09 and H1 SIVs in pigs (Kyriakis et al. 2010), detection of antibodies against SIVs was not performed in this study. Serological surveillance for influenza viruses was, therefore, conducted in Cambodian pigs for the detection of antibodies against human H1N1, human H3N2, human A(H1N1)pdm09 and avian H5N1viruses.
7.2 Materials and methods

7.2.1 Serum samples

A total of 1147 serum samples collected from pigs in Cambodia between 2006 and 2010 were tested for influenza viruses at the Institut Pasteur in Cambodia (IPC) (Figure 7.1).

![Diagram](image)

**Figure 7.1.** Source of samples and the testing regime.

The sera comprised stored serum specimens from a repository at the National Veterinary Research Institute (NaVRI) of Cambodia and samples collected by the IPC from a slaughterhouse in Phnom Penh. All samples were tested by HI assays for detection of antibodies against seasonal human H1N1 and H3N2 influenza viruses. The 372 serum specimens collected from 2009 to 2010 were additionally tested for
the A(H1N1)pdm09 virus. Also, 150 samples were randomly selected and tested further for antibodies against avian influenza H5N1 virus. Data on gender, province of origin and date of sample collection were recorded for all 1147 animals tested. Data on age were recorded except for animals sampled in the slaughterhouse.

### 7.2.2 Reference viruses

Human and avian influenza A viruses circulating in Cambodia during the year of sampling were chosen as reference viruses in this study (Table 7.1). Each sample was tested against the reference strain per subtype from the year of sampling of that specific sample. All reference viruses were extracted from the repository of the Virology Unit, National Influenza Centre at the IPC. Assays using H5N1 virus were conducted under biosafety level 3 conditions.
### Table 7.1. Reference viruses used in the serological tests.

<table>
<thead>
<tr>
<th>Influenza virus</th>
<th>Year of sampling</th>
<th>Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/New Caledonia/20/99 (H1N1)</td>
<td>2006-2007</td>
<td>HI</td>
</tr>
<tr>
<td>A/Wisconsin/67/2005 (H3N2)</td>
<td>2006-2007</td>
<td>HI</td>
</tr>
<tr>
<td>A/Brisbane/59/2007 (H1N1)</td>
<td>2008</td>
<td>HI</td>
</tr>
<tr>
<td>A/Brisbane/10/2007 (H3N2)</td>
<td>2008</td>
<td>HI</td>
</tr>
<tr>
<td>A/Brisbane/59/2007 (H1N1)</td>
<td>2009-2010</td>
<td>HI</td>
</tr>
<tr>
<td>A/Perth/16/2009 (H3N2)</td>
<td>2009-2010</td>
<td>HI</td>
</tr>
<tr>
<td>A/California/7/2009 (H1N1)</td>
<td>2009-2010</td>
<td>HI</td>
</tr>
<tr>
<td>A/Cambodia/Q0321176/2006 (H5N1)</td>
<td>2006</td>
<td>HI,MN</td>
</tr>
<tr>
<td>A/Cambodia/S1211394/2008 (H5N1)</td>
<td>2008</td>
<td>HI,MN</td>
</tr>
<tr>
<td>A/Cambodia/T1218159/2009 (H5N1)</td>
<td>2009</td>
<td>HI,MN</td>
</tr>
<tr>
<td>A/Cambodia/U0417030/2010 (H5N1)</td>
<td>2010</td>
<td>HI,MN</td>
</tr>
</tbody>
</table>

### 7.2.3 HI assay

A total of 1147 serum samples were tested by the HI test, which has been commonly used to detect the presence of antibody to the HA of influenza viruses in animal and human sera. Before testing, the samples were treated with receptor-destroying enzyme (RDE; Denka Seiken Co. Ltd., Tokyo, Japan) to remove non-specific haemagglutination inhibitors, incubated in a water bath at 37°C overnight and heated in a water bath at 56°C for 30 minutes to inactivate RDE. The RDE-treated sera were then mixed with 1 drop of 2% red blood cells (RBCs) diluted to 1:10 with 0.85% NaCl solution. The RDE-treated sera and RBCs were thoroughly mixed together by hand shaking and kept in a refrigerator for 1 hour.
HI tests were performed using 96-well polystyrene, microtitre plates. In each test, positive and negative serum controls were included. Briefly, 50 µl of PBS was added from rows B to H prior to addition of 50 µl of RDE-treated sera from rows A to H. Serial two-fold dilutions were made by transferring 50 µl amounts from the first row to successive rows and in the final row 50 µl was discarded. Antigen containing four HA units/50 µl of the reference virus was then added to each well and the plates were incubated at room temperature for 15 minutes. Fifty microlitres of RBCs were then added to each well.

When using influenza viruses of avian origin, horse blood cells are preferred as they express only receptors to ‘avian-type’ antigens. For seasonal influenza, the red blood cell type that gave the clearest agglutination with each virus was selected. For H1N1 and H3N2 testing, 0.75% guinea pig RBCs was used. For the A(H1N1)pdm09 virus, 0.5% Turkey RBCs were used and for H5N1 virus, 0.5% horse RBCs were utilized (WHO 2011). The plates were incubated at room temperature for 1 hour. The HI titre was expressed as the highest reciprocal serum dilution that completely inhibited the haemagglutination of 4 HA units of the virus. Considering the previous studies (Chambers et al. 1991; Olsen et al. 2000), HI titres of 1:40 and higher were regarded as positive.

7.2.4 Microneutralisation assay

The MN test that detects HA subtype-specific antibody is frequently used in parallel with the HI assay for avian influenza virus serology in mammalian specimens (Jung et al. 2007). One hundred and fifty serum samples collected from Cambodian pigs
and randomly selected were tested for avian influenza antibodies by the MN assay in
the BSL3 laboratory of the Virology Unit at the IPC. The MN assay was performed
only when the HI titre was ≥20. Briefly, all sera that were already treated with RDE
were also heat inactivated at 56°C for 30 minutes. For standard MN assays, 100
tissue culture infectious dose 50 (100 TCID50) of the avian influenza virus, with
serial two fold dilutions of each serum sample (starting from 1:10), were incubated
for one hour at room temperature, followed by inoculation of the virus-antibody
mixture onto MDCK cells. Cell monolayers were incubated and examined daily for
cytopathic effects for 3–4 days. Determining endpoint neutralizing antibody titres
was carried out in four wells per dilution. The neutralising titre was defined as the
reciprocal of the highest dilution of serum at which the infectivity of 100 TCID50 of
an H5N1 virus for MDCK cells was completely neutralised in 50% of the wells. The
titre was calculated by the Reed and Muench method (Reed and Muench 1938). A
seropositive specimen to avian H5 virus was defined by HI and MN titres against
H5N1 virus ≥40 (Olsen et al. 2000).

7.2.5 Data analysis
The seroprevalence was calculated along with the 95% confidence intervals using
the exact binomial method (Ross 2003). The mean ± SD of HI antibody titres was
calculated. The seroprevalence rates were compared between years, age and
province. Statistical analyses were performed in SPSS version17 (SPSS Inc.,
Chicago, IL, USA). Seropositivity to human influenza viruses between two age
groups was also compared by the two-sided Fisher’s exact analysis. Animals for
which age was not recorded were excluded from the age analysis.
7.3 Results

The seroprevalence rates to each human influenza A serotype and to avian H5N1 virus were calculated. The overall seroprevalence to human influenza A viruses during the study period was 14.9%. A(H1N1)pdm09 virus was the dominant (23.1%) subtype detected in pigs by serology followed by the seasonal H1N1 virus (17.3%) and the H3N2 subtype (9.9%). Antibodies against more than one subtype were detected in 132 individual pigs. Seroprevalence to seasonal H1N1 virus ranged between 2.7% in 2007 and as high as 46.5% in 2008 (Table 7.2). The prevalence of anti-H3 antibodies in pig sera varied between 0% in 2007 and 33.8% in 2008. Serology to A(H1N1)pdm09 tested positive only in samples collected in 2010. None of the tested sera showed positive antibodies to H5N1 virus.

Table 7.2. Annual seroprevalence to each influenza A virus subtypes tested (n = 1147).

<table>
<thead>
<tr>
<th>Year</th>
<th>No. of sera</th>
<th>H1 (%) 95% CI</th>
<th>H3 (%) 95% CI</th>
<th>A(H1N1)pdm09 (%) 95% CI</th>
<th>H5 (%) 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td>393</td>
<td>5.6 3.5-8.4</td>
<td>2.3 1.1-4.3</td>
<td>NT</td>
<td>0a 0.0-12.8</td>
</tr>
<tr>
<td>2007</td>
<td>113</td>
<td>2.7 0.6-7.6</td>
<td>0.0 0.0-3.2</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>2008</td>
<td>269</td>
<td>46.5 40.4-52.6</td>
<td>33.8 28.2-39.8</td>
<td>NT</td>
<td>0a 0.0-7.0</td>
</tr>
<tr>
<td>2009</td>
<td>36</td>
<td>19.4 8.2-36.0</td>
<td>13.9 4.7-29.5</td>
<td>0.0</td>
<td>0a 0.0-9.7</td>
</tr>
<tr>
<td>2010</td>
<td>336</td>
<td>12.5 9.2-16.5</td>
<td>2.4 1.0-4.6</td>
<td>25.6</td>
<td>21.0-30.6</td>
</tr>
</tbody>
</table>

* Only a subset of the samples were tested for antibodies against H5.

NT: not tested
The overall seroprevalence to the viruses tested was notably low in 2006 and 2007 and peaked in 2008 before decreasing in 2009 and 2010 when the peak of A(H1N1)pdm09 was observed. The range and mean±SD of antibody titres to H1N1, H3N2 and A(H1N1)pdm09 viruses are shown in Table 7.3.

**Table 7.3.** HI titres to three different influenza subtypes.

<table>
<thead>
<tr>
<th>Antibody titers</th>
<th>Different influenza subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H1N1</td>
</tr>
<tr>
<td>Range</td>
<td>0–320</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>16.2 ± 29.4</td>
</tr>
<tr>
<td>No. of sera tested</td>
<td>1147</td>
</tr>
</tbody>
</table>

The seroprevalence of H1N1, H3N2 and A(H1N1)pdm09 viruses was compared between pigs ≤4 months old and those >4 months old (Table 7.4). The seroprevalence of H1N1 and H3N2 viruses was higher in the younger age group (≤4 months old), but the difference was not significant (P >0.05).
### Table 7.4. Seroprevalence to H1N1, H3N2 viruses in two age groups (n= 538).

<table>
<thead>
<tr>
<th>Period</th>
<th>Seropositivity: number positive/number tested (%)</th>
<th>H1N1 95%CI</th>
<th>H3N2 95%CI</th>
<th>A(H1N1)pdm09 95%CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-pandemic</td>
<td>150/775 (19.4)</td>
<td>16.6-22.3</td>
<td>100/775 (12.9)</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td>7/55 (12.7)</td>
<td>5.3-24.5</td>
<td>5/55 (9.1)</td>
<td>0/55 (0.0)</td>
</tr>
<tr>
<td></td>
<td>42/317 (13.2)</td>
<td>9.7-17.5</td>
<td>8/317 (2.5)</td>
<td>86/317 (27.1)</td>
</tr>
<tr>
<td>Post-pandemic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>0.03</strong></td>
<td><strong>0.0000012</strong></td>
<td><strong>0.000003</strong></td>
<td></td>
</tr>
</tbody>
</table>

*Statistical analysis for differences of test results between different periods; P < 0.05 is considered statistically significant

* Determined by Chi-square analysis

** Determined by Fisher’s exact test

NT: not tested

The seroprevalence by province of origin of the animals (Banteay Meanchey, Kampong Cham, Kampong Speu, Kampot, Kandal, Prey Veng, Pursat, Svay Rieng and Takeo provinces) is shown in Figure 7.2. Evidence of seasonal H1N1 and H3N2 influenza viruses’ circulation in pigs was found from eight of the nine provinces from which pigs were sampled (88.9%). All sera originating from Kampot province (n = 19) were seronegative to all subtypes. The highest seroprevalence to seasonal H1N1 virus was 52.2% in Banteay Meanchey province. The highest seroprevalence to H3N2 virus was 33.3% in Pursat province and the lowest (0%) in Kampong Speu and Kampot provinces. Samples collected from pigs originating from four provinces (Kandal, Prey Veng, Svay Rieng and Takeo) were tested for A(H1N1)pdm09 virus by serology after the introduction of the virus in country and positive results were
found in all the four provinces, ranging from 8% in Takeo to 35% in Kandal province.

\[\text{Figure 7.2. Seroprevalences against H1N1, H3N2 and A(H1N1)pdm09 viruses in various provinces in Cambodia.}\]

\[\text{7.4 Discussion}\]

Pigs are susceptible to infection with influenza viruses from mammalian and avian origins (Moreno et al. 2011) and play an important part in the ecology of influenza A viruses, being a potential source for human pandemic influenza viruses with serious
public health implications (Lipatov et al. 2008). According to previous studies, human H1N1 and H3N2 viruses are frequently transmitted to pigs through reverse zoonosis; however, they do not show long-term persistence in pig populations (Van Reeth 2007). Nevertheless, the genes of human viruses may persist after reassortment with one or more influenza viruses in pigs (Moreno et al. 2011). Such circumstances could lead to generation of reassortant viruses with increased cross-species transmissibility, pathogenicity and lethality, which could cause a human influenza pandemic.

In this study, sera from pigs collected in Cambodia between 2006 and 2010 were tested for antibodies to influenza A (human H1N1, human H3N2, A(H1N1)pdm09 and avian influenza (H5N1) viruses. No serological tests to detect SIVs were performed. Indeed, SIVs have never been isolated in Cambodia and only rarely in surrounding countries of the region. It should be noted that the HI tests fail to differentiate between A(H1N1)pdm09 and SIVs owing to serologic cross-reactivity in pigs (Kyriakis et al. 2010). The average seroprevalence against the human influenza A viruses tested was 14.9% during the study period. This result is different to those reported in semi-commercial farms in Vietnam (3.1%) and industrial farms in China (61.4%) (Liu et al. 2011; Trevennec et al. 2011).

The highest seroprevalence detected in Cambodian pigs was against the A(H1N1)pdm09 influenza virus followed by the seasonal H1 and the H3 subtypes, respectively. The results also showed evidence that some pigs were exposed to more than one human virus during their short lives. The high levels of A(H1N1)pdm09
virus infections in Cambodian pigs suggest that this strain was widely circulating in the pig population as described in other countries, including those located in Asia (Song et al. 2010; Sreta et al. 2010; Forgie et al. 2011). The potential for concurrent multiple infections with human influenza viruses in pigs needs to be emphasised as it facilitates the opportunity for the generation of new pathogenic variants in pigs through reassortment events, which might then facilitate transmission to humans (Yoon and Janke 2002; Zhu et al. 2011). Moreover, dual infection with A(H1N1)pdm09 and H3N2 viruses in humans has been documented in Cambodia (Myers et al. 2011).

H5N1 virus has been isolated from pigs on a few occasions in Indonesia and China with evidence of pig-to-pig transmission in Indonesia (Zhu et al. 2008; Takano et al. 2009; Nidom et al. 2010), but this virus is still generally considered poorly transmissible to swine (Lipatov et al. 2008). Antibodies against avian H5 influenza virus were not detected in this study. The findings of the current study, therefore, suggest a low risk of reassortment between avian H5N1 and A(H1N1)pdm09 viruses. Nevertheless, the number of samples tested was limited and the circulation of H5N1 virus in poultry is seasonal and geographically restricted to some provinces. Therefore, H5N1 seroconversions may not have been detected.

The overall seroprevalence of each influenza virus subtype detected in pigs follows the human seasonal serotype pattern that was seen in Cambodia during these recent years (IPC unpublished data). The similarity of the seroprevalence in pigs and humans suggests a possible human-to-swine influenza virus transmission in Cambodia. Moreover, the results from our study demonstrated a high seroprevalence
to the A(H1N1)pdm09 virus during the post-pandemic period. The situation in the swine population mimics that described for humans where the A(H1N1)pdm09 virus progressively replaced the seasonal H1N1 influenza virus and became the predominant circulating subtype in humans. No samples seropositive against the A(H1N1)pdm09 virus were found during 2009, but the community-level transmission of A(H1N1)pdm09 viruses in human population in Cambodia started only in August 2009 suggesting that only a short delay was required for the transmission from humans-to-swine. These data also suggest that these positive A(H1N1)pdm09 tests were not a result of cross-reactivity with potentially circulating H1N1 SIVs, as no samples prior to 2010 were seropositive using this test.

Pigs were categorised into two age groups (≤4 months old and >4 months old) as maternal antibodies to influenza viruses can persist for up to 16 weeks (Easterday and Van Reeth 1999). Few data on age were available for the pigs that tested positive by serology to A(H1N1)pdm09 virus and this explains why comparison by age groups was not possible. No statistically significant difference in the seroprevalence between the two age groups was detected. This may have resulted from interference by maternal antibodies in the younger age group (≤4 months old), while previous exposure to influenza viruses could explain the antibody status in the older age group (>4 months old). However, considering the results should be interpreted with care. In this study the seroprevalence rates in pigs sampled in farms were not compared with those sampled in slaughterhouses because pigs sent to abattoirs are mostly 10–12 months old, which are older than those living on farms (Duong et al. 2011).
In eight of nine provinces, evidence of H1 and H3 influenza virus infections were found. Serology was surprisingly negative in pigs originating from Kampot, but the low number of samples collected means that this result should be interpreted with caution. A(H1N1)pdm09 virus infections were detected in pigs originating from all four provinces sampled after the beginning of the pandemic. This suggests extensive circulation of human influenza virus infections in pigs across Cambodia, although, without characterisation of the viruses themselves, it cannot be determined whether there is ongoing circulation of these viruses in swine populations nationally or whether these infections were the result of discrete introductions from human populations with limited onward spread in pigs. The provinces with high seroprevalences should be investigated further in terms of human influenza cases and human-to-pig interface. Serum samples were mostly collected from a slaughterhouse in Phnom Penh. Pigs are usually slaughtered shortly after arriving in the capital (generally within 24 hours), which does not give sufficient time for a pig to seroconvert following a contamination event that may occur at the slaughterhouse. Thus, influenza virus contaminations were presumed to have occurred in farms.

Some experimental studies have shown that the HI tests are sufficient to differentiate antibodies to H1N1, H3N2 and H1N2 SIV subtypes in European swine (Reeth et al. 2004; Van Reeth et al. 2006). However, Kyriakis et al. (2010) hypothesized that if pigs had been previously infected with, or vaccinated against, European SIVs, they would frequently have serologic cross-reactivity to the A(H1N1)pdm09 virus and related North American SIVs. Hence, sera from pigs either infected or vaccinated with SIVs could have cross-reactive HI antibodies to the A(H1N1)pdm09 virus. However, no autogenous or commercial swine influenza vaccines are believed to
have been used in the Cambodian swine industry. In addition, no SIVs have been previously isolated in Cambodia. Although a definitive answer would have required to also test each sera for the detection of anti-nucleoprotein antibodies (specific for influenza A), this, along with the lack of positive samples from before 2010, makes the chance of cross-reactivity to A(H1N1)pdm09 in this study unlikely.

As no routine or systematic surveillance for influenza A in Cambodian pigs has been carried out previously, this study was initiated by the IPC through collaboration with the NaVRI. Given the limited resources in establishing nationwide surveillance for influenza in pigs, it was considered that slaughter pigs are suitable sentinels to determine the presence of influenza A viruses. Archival samples from NaVRI that were collected from pigs originating from farms in Kampong Cham, Kampong Speu and Takeo provinces were also included to increase the power of the statistical analysis. The results shown here, therefore, do not perfectly represent the entire pig population in Cambodia because of sampling bias. The majority of samples (682 of 1147) were taken from pigs of marketable weight at the abattoir, which makes the extrapolation to the whole pig population in Cambodia difficult. To illustrate, pigs in Cambodia are generally slaughtered at the age of 10–12 months, thus an overrepresentation of pigs at the abattoir cannot be excluded. However, these results are useful to identify the dominant influenza strains in pigs in the country and to emphasise the urgent need to implement a well-designed surveillance system for influenza A in the Cambodian swine population.

A more systematic surveillance study needs to be developed and applied for the investigation of influenza A viruses in pig populations in Cambodia. Furthermore,
studies to collect and characterise viruses as well as using molecular techniques to detect, monitor and evaluate the persistence of circulating strains of influenza viruses in pigs farms rather than in abattoirs, where only apparently healthy animals are slaughtered, are recommended to identify their future evolution and ensure early detection of potentially pandemic strains. In addition, molecular surveillance is required for the study of genetic components of influenza viruses to closely monitor their characterisation, their extent of reassortment and their potential impact on public health. Participation at the community level needs to be incorporated into the existing surveillance for influenza viruses in Cambodian pigs to enhance the sensitivity of detecting influenza cases in swine.

In conclusion, this study provides the first data on sustained human influenza virus infections in pigs in Cambodia. Serological surveillance results indicated that seasonal H1, H3 and A(H1N1)pdm09 subtypes were common in Cambodian pigs and probably resulted from extensive transmission of influenza A virus from humans back to pigs. On the other hand, infection with the H5 subtype was not detected. Serological investigation of influenza viruses may provide useful information for surveillance of novel influenza viruses in pigs.

In the next Chapter the serological data are analysed further 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.
Chapter 8: Epidemiological Analysis of Influenza A Infection in Cambodian Pigs

8.1 Introduction

Influenza A viruses are members of the family Orthomyxoviridae and are categorised into different subtypes on the basis of the antigenic properties of envelope glycoproteins, namely haemagglutinin and neuraminidase. 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 might 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). Pig management in Cambodia is primarily traditional, and 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, no influenza viruses have been isolated from Cambodian pigs. However, a 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 generalised 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. Strategies are also proposed 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.

8.2 Materials and methods

8.2.1 Study population

Existing laboratory data (n = 1147) on the serological evidence of infection of pigs from Cambodia with human influenza viruses were examined. The data were sourced from the NaVRI of Cambodia and the 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 (Figure 8.1).
Figure 8.1. Map of Cambodia showing administrative units divided into 24 provinces with geographical variations of number of sampling occasions (legend) and number of samples collected in various provinces (number below province name).

Each sample was tested by 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 densities of pigs, humans and poultry and the road density. The last three factors represented risk factors for highly pathogenic avian influenza (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 of Chetra and Bourn (2009) (see Figure 8.2). 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 Arc-GIS 9 (Environmental System Research Institute, Redlands, CA, USA).
Figure 8.2. Poultry and pig population densities by Cambodian province in 2006-2008 (Chetra and Bourn 2009).
8.2.2 Statistical analyses

The relationship between seroprevalence to seasonal H1N1 and H3N2 influenza viruses and the selected factors was examined using generalised 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 ≥ 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 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.

8.3 Results

8.3.1 Seroprevalence by sampling occasion

The seroprevalence against seasonal H1N1 and H3N2 influenza viruses for each sampling occasion is summarised in Table 8.1. 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%.
Table 8.1 Seroprevalence of seasonal H1N1 and H3N2 antibodies amongst sampling occasions.

<table>
<thead>
<tr>
<th>Occasion no.</th>
<th>Province</th>
<th>Month</th>
<th>Year</th>
<th>Source</th>
<th>Location</th>
<th>n</th>
<th>% Seroprevalence (95% CI)</th>
<th>H1N1 subtype</th>
<th>H3N2 subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>5,6,7,8</td>
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<td>Abattoir</td>
<td>23</td>
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<td>13.0 (2.8-33.6)</td>
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<td>NaVRI</td>
<td>Farm</td>
<td>333</td>
<td>4.2 (2.3-7.0)</td>
<td>2.7 (1.2-5.1)</td>
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<td>7</td>
<td>2006</td>
<td>NaVRI</td>
<td>Farm</td>
<td>60</td>
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<td>12</td>
<td>2007</td>
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<td>43.3 (33.3-53.7)</td>
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<td>IPC</td>
<td>Abattoir</td>
<td>60</td>
<td>8.3 (2.8-18.4)</td>
<td>3.3 (0.4-11.5)</td>
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<td>Prey Veng</td>
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<td>Abattoir</td>
<td>9</td>
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<td>9</td>
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<td>IPC</td>
<td>Abattoir</td>
<td>157</td>
<td>12.7 (8.0-19.0)</td>
<td>2.6 (0.7-6.4)</td>
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<td>10</td>
<td>Pursat</td>
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<td>IPC</td>
<td>Abattoir</td>
<td>27</td>
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<td>Abattoir</td>
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<td>5,6,7,8</td>
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<td>Abattoir</td>
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<td>2010</td>
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<td>12</td>
<td>2007</td>
<td>IPC</td>
<td>Abattoir</td>
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<td>2009</td>
<td>NaVRI</td>
<td>Farm</td>
<td>36</td>
<td>13.9 (4.7-29.5)</td>
<td>19.4 (8.2-36.0)</td>
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<td>17</td>
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<td>8,11</td>
<td>2010</td>
<td>NaVRI</td>
<td>Farm</td>
<td>25</td>
<td>0.0 (0.0-13.7)</td>
<td>0.0 (0.0-13.7)</td>
<td></td>
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<td>18</td>
<td>Takeo</td>
<td>2</td>
<td>2010</td>
<td>NaVRI</td>
<td>Farm</td>
<td>11</td>
<td>18.2 (2.3-51.8)</td>
<td>0.0 (0.0-28.5)</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Takeo</td>
<td>11</td>
<td>2010</td>
<td>IPC</td>
<td>Abattoir</td>
<td>28</td>
<td>21.4 (8.3-41.0)</td>
<td>0.0 (0.0-12.3)</td>
<td></td>
</tr>
</tbody>
</table>
8.3.2 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 8.2). The two variables included in the model as fixed effects were human density and pig density because it was expected that these two variables would have the strongest impact on the pig seroprevalence, despite their PCC being superior to 0.28 (Table 8.2).

**Table 8.2.** The Pearson correlation coefficients calculated for selecting fixed effects in the models.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Human density</th>
<th>Pig density</th>
<th>Poultry density</th>
<th>Road density</th>
</tr>
</thead>
<tbody>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pig density</td>
<td>0.38</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Poultry density</td>
<td>0.54</td>
<td>0.67</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>Road density</td>
<td>0.84</td>
<td>0.31</td>
<td>0.20</td>
<td>1.0</td>
</tr>
</tbody>
</table>

8.3.3 The models used for investigating factors influencing seroprevalence

According to the 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 8.3 and 8.4, respectively. The best-supported models for H1N1 and the top three best-supported models for H3N2 included human population density and pig population density. 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 demonstrated that year mostly accounted for the random part (Tables 8.3 and 8.4).

**Table 8.3.** Summary of the best-supported models fitted to estimate variations in seroprevalence against seasonal H1N1 influenza viruses in pigs in Cambodia.

| Model | AIC  | k | Random effecta | Estimate  | Pr(>|z|)b |
|-------|------|---|----------------|-----------|----------|
|       |      |   |                | 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         | 0.0008    | -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        |

aVariance estimation
Table 8.4. Summary of the best-supported models fitted to estimate variations in seroprevalence against seasonal H3N2 influenza viruses in pigs in Cambodia.

| Model | AIC      | k | Random effect | Estimate Human density | Estimate Pig density | Pr(>|z|) | Pr(>|z|) Pig density |
|-------|----------|---|---------------|------------------------|----------------------|---------|---------------------|
|       |          |   |               |                        |                      |         |                     |
| 1     | 47.52    | 3 | 3.149         | 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 **| 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   |                     |

*Variance estimation

bSignificant codes: 0 ‘***’, 0.001 ‘**’, 0.01 ‘*’, 0.05 ‘.’

8.3.4 Statistical analyses

No significant association between seroprevalence of H1N1 and the population density of humans or pigs was found (Table 8.3). Seroprevalence of H3N2 was positively associated with density of the human population, whereas it was negatively related to the pig population density (Table 8.4).
8.4 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 tests 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).

However no autogenous or commercial vaccines against swine influenza have been reported to 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 Pasteurin 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, it is speculated 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 as the statistical analysis may have been compromised by a level of spatial bias arising from the non-randomly collected samples. Some unexpected results may thus be linked to the sampling strategy. To illustrate, the data analysed represented combined serological results of 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 at a farm level. The underlying variations in pig farm management within the provinces, such as husbandry practices, biosecurity and hygiene, may also interfere with the results. Therefore, this study has demonstrated the limitations of analysing risk factors 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 (Figure 8.1) 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. It is recommended that sampling methods should be improved and metadata relating to the animals sampled should be systematically collected at farms or abattoirs for future epidemiological studies in order to effectively identify and quantify the drivers of influenza virus infection in pigs.
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. 2012b; 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.

Two main approaches for 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. Secondly, surveillance should be based on the assessment of antibody levels. Defining a baseline level of antibodies may help the early detection of influenza outbreaks in target pig populations by recognising an increased seroprevalence. The 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). Importantly, antigens for HI tests should be regularly compared with those from circulating strains in a given country or region (Sreta et al. 2013). 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.

This study demonstrated an epidemiological analysis of influenza A virus transmission in Cambodian pigs that showed 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 and other countries where funds are limited has thus been emphasised. To further refine these surveillance strategies, a better understanding of human–pig–avian interfaces and detailed pig trade in the country are needed. Moreover, socioeconomic studies are required to adapt the surveillance schemes to the local context.
Chapter 9: General discussion

9.1 Introduction
Influenza A viruses infect a large variety of animal species including humans, pigs, horses, sea mammals and birds (Alexander, 1982; Webster et al. 1992). Pigs are an important host in influenza virus ecology due to their dual susceptibility to infections with both avian and human influenza A viruses. Furthermore, pig husbandry and management practices allow an opportunity for regular close contact between pigs and humans or pigs and birds that could potentially result in virus transmission among these species (Brown 2000). Recently, the 2009 pandemic has rekindled interest in pigs as mixing vessels for influenza viruses as the A(H1N1)pdm09 virus is a reassortant virus with genes from two distinct lineages including North American triple reassortant and European avian-like H1N1 subtype that have been circulating in pigs for a long time (Garten et al. 2009).

Following a rapid spread of A(H1N1)pdm09 virus in human populations, pigs worldwide became infected as a result of exposure to infected humans. Interestingly, a number of cases in Europe and other parts of the world suggested virus dissemination through pig-to-pig transmission rather than through contact with infected humans (Brookes and Brown 2011). The maintenance of the A(H1N1)pdm09 virus in pigs provides a further opportunity for second generation reassortment viruses through coinfection with other endemic swine influenza virus strains (Brookes and Brown 2011). There have been reassortment of A(H1N1)pdm09 viruses reported in pigs in several countries (Vijaykrishna et
al.2010; Ducatez et al. 2011; Howard et al. 2011; Moreno et al. 2011; Starick et al. 2011; Tremblay et al. 2011; Fan et al. 2012; Liu et al. 2012a). Thus, epidemiological studies are essential to understand the situation of influenza A viruses in pig populations and to develop public health strategies for disease prevention.

In the past 20-25 years, livestock production in Thailand has shifted from backyard animals and integrated cropping-livestock farming systems to industrial livestock farming enterprises (Riethmuller and Chalermpao 2002; Charoensook et al. 2013). However, biosecurity in rural communities in Thailand is often suboptimal and local smallholders usually raise domestic pigs that cohabit with free-range chickens. In Cambodia, the livestock sector is dominated by smallholders (Huynh et al. 2007). Cambodian pigs are usually raised with low biosecurity, facilitating opportunities for close contact between humans and pigs. Prior to this thesis, very limited information was available on the epidemiology of influenza A viruses in pigs in Thailand and Cambodia. The research studies reported in this thesis were conducted in order to gain a better understanding of the disease situation, as well as to define disease control, surveillance strategies and public health campaigns suitable for adoption in the local context.

9.2 Influenza situation in pigs in Thailand and Cambodia

In Thailand, virus isolation was attempted from pigs on commercial farms (Chapter 3) and in small farms (Chapter 6). No positive samples were recovered from pigs in the small farms in Mukdahan provinces. In contrast, eight influenza A viruses were isolated from weaning pigs in commercial farms in Chonburi and Chachoengsao provinces. Two viruses isolated were found to be reassortant H3N2 viruses that
consisted of the HA and NA genes from a swine H3N2 virus and other internal genes from the A(H1N1)pdm09 virus. The remaining six viruses isolated were all A(H1N1)pdm09. These findings suggest that the A(H1N1)pdm09 virus has become established in the Thai pig population and provides opportunities for second generation reassortment with endemic swine influenza virus strains. Thus it is crucial to closely monitor the genetic evolution of the A(H1N1)pdm09 virus in the Thai pig population.

In Cambodia, no influenza A viruses in pigs had previously been detected (Institut Pasteur in Cambodia, unpublished data). However, the results reported in Chapter 7 demonstrated sustained transmission of human influenza virus infections in the Cambodian pig population as the seroprevalence of seasonal H1, H3 and A(H1N1)pdm09 subtypes in Cambodian pigs was high. The evidence of extensive transmission of influenza A viruses from humans to pigs in Cambodia has emphasised the urgent need to develop an effective surveillance system. Further studies on genetic characterisations of influenza A viruses circulating in the Cambodian pig population are recommended in order to ensure early detection of potentially pandemic strains.

9.3 Disease control and prevention

The pig husbandry practices adopted in rural Thailand may increase the potential for virus spread on farms (Chapter 4). A number of husbandry characteristics that could pose significant challenges to the control and prevention of influenza A viruses in pigs were observed. Firstly, the intermingling of multiple species e.g. free-ranging chickens was commonly found on most studied farms. Mammalian pets on pig farms
may serve as potential carriers to spread the viruses to pigs (Suriya et al. 2008). Keeping pigs close to other species should therefore be discouraged in order to decrease the risk of cross-species transmission and the possibility of reassortment of influenza A viruses. Secondly, pigs were raised in traditional pig pens that could facilitate the spread of airborne pathogens through close contact between pigs from different pens and also may favour the survival and transmission of influenza A viruses in the environment. For cleaning pens, the use of readily available products in households that are able to kill the A(H1N1)pdm09 virus, such as 1% bleach, 10% vinegar and 0.01% washing up liquid, should be promoted (Greatorex et al. 2010).

No restrictions on visitors or biosecurity protocols were applied on most studied farms. Using manure as field fertilizer, hiring boars from outside the piggery and trading activities in the local community were regularly conducted. Importantly, this could contribute to the potential spread of influenza viruses in the region. Therefore, limiting visits and improving biosecurity practices should be adopted as these play an important role in preventing virus introduction and lowering the chance of virus dissemination between farms, as well as reducing the likely introduction of other infectious diseases. Lastly, identification of pigs was not implemented on most farms and this could compromise disease control and surveillance efforts. On-farm identification should thus be encouraged to ensure traceability of animals. With animal identification, if an outbreak occurred, control and eradication processes could be promptly implemented so that disease spread and production losses could be reduced in a timely manner (Ammendrup and Barcos, 2006).
A policy of annual influenza vaccination of swine workers may help decrease the possibility of reassortment events occurring (Myers et al. 2007). During pandemics, agricultural workers should be included in influenza surveillance efforts and be considered as a priority group for annual influenza vaccination and antivirals (Gray et al. 2007). In particular, the A(H1N1)pdm09 virus could become established in pig populations following primary contact with infected humans (Brookes and Brown, 2011). However, most farmers had never been vaccinated against the A(H1N1)pdm09 virus (Chapter 4). In addition, the study on KAPs of pig smallholders in rural Thailand found that most farmers had limited knowledge of influenza A in pigs (Chapter 5). Public health campaigns for preventing virus transmission between pigs and humans should therefore be developed to provide farmers in rural Thailand with the correct knowledge and assist in changing their behaviours into appropriate practices such as avoiding contact with pigs and wearing personal protective equipment.

9.4 Surveillance prospects

Surveillance strategies in the context of weak infrastructure have been outlined (Chapter 8). Since it would be impossible to conduct nationwide surveillance in low-income countries due to limited financial and technical resources, developing a targeted surveillance system within the concept of risk-based surveillance would be appropriate as it requires a smaller sample size and utilizes fewer resources. This could be accomplished by focusing on subpopulations that are anticipated to have a higher risk of infection. For example, weaning pigs should be targeted for virus detection to increase the chances of isolating influenza A viruses (Takemae et al. 2011). Pigs on at-risk farms that are epidemiologically linked to cases of influenza-
like illness in pigs or humans should also be targeted for sampling. High-risk places such as markets and slaughterhouses, where pigs from numerous sources commingle, would be suitable 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.

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 with increased pathogenicity back to humans (Liu et al. 2012b; Trevennec et al. 2012). However, due to the low isolation rates of influenza viruses from pigs in East and South-East Asia, a large number of biological samples would need to be collected in order to successfully detect the viruses (Trevennec et al. 2011). Furthermore, the chance of detecting influenza A viruses in pigs is limited given the short period of virus shedding. The practical use of virological monitoring is therefore impeded by its high cost, time consumption, high technical demand for laboratory capacity and the need for skilled workers.

Although serological data are often thought to be of limited value, as they can 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. In resource-limited countries, serological testing for disease surveillance
is therefore appropriate. To monitor long-term trends and to detect disease emergence, assessment of antibody levels should be used by computing a baseline seroprevalence of influenza A viruses in pig populations. 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. It is important to note that specimens should be obtained from both healthy and clinically ill pigs as subclinical infections can occur. Moreover, a good passive network and education campaign should be developed in order to encourage farmers to report respiratory cases. It is also recommended that an active surveillance system is established by organising a network of sentinel farms in high-risk areas.

9.5 Limitations

A potential limitation of the current study was selection bias. In Chapter 3, five commercial pig farms were selected based on farmers’ collaboration. Moreover, the studies in Chapters 4, 5 and 6 were conducted in one rural community that may not necessarily represent the general population. Furthermore, the results of the pigs sampled in Chapters 6 and 7 may not be representative of the pig population. Due to limited resources, the nasal swab and serum samples reported in Chapter 6 were targeted to a population with an increased probability of testing positive. Specifically only piglets were targeted for influenza virus detection and only sows were selected as subjects for antibody detection to influenza A viruses. Consequently it is difficult to extrapolate the current findings to the indigenous pig population in the community. However if infection was present, the targeted sampling used would have had a greater chance of virus detection than true random sampling. In Chapter 7, the majority of samples were taken from slaughtered pigs, while the remaining
samples were collected directly on-farm. Thus, an overrepresentation of pigs with marketable weight cannot be excluded.

9.6 Recommendations

Based on the findings in this thesis, the following recommendations are proposed.

- **Data collection and analysis**

Metadata relating to the animals sampled should be recorded including animal age, farm type, farm husbandry practices and farm location (at least the village and district of origin of the animal). This would provide accurate spatial data of the animal origin and informative data on potential risk factors for subsequent epidemiological investigations. Regarding KAPs, a multivariate model should be used to statistically analyse the findings in order to determine significant predictors of KAPs in farmers such as age and education for development of suitable intervention strategies.

- **Laboratory procedures**

It is recommended that virus isolation should be performed in local laboratories in order to prevent loss of virus infectivity relating to the freeze-thaw process or through poor cold chain management. Clinical specimens for viral isolation should be placed on ice packs and transported to the laboratory promptly after sample collection.

- **Surveillance strategies**

A more systematic surveillance study needs to be developed and applied for the investigation of influenza A viruses in pig populations in this region. Participation at
the community level needs to be incorporated into the existing surveillance for influenza viruses in pigs to enhance the sensitivity of detecting influenza cases in swine. To investigate the diversity of influenza viruses at the human–pig interface, influenza virus surveillance in both pig and human populations should be carried out (Terebuh et al. 2010). Additionally, closely monitoring pig farmers as potential sentinels may be appropriate for early detection of novel influenza viruses originating from pig herds (Olsen et al. 2002).

9.7 Conclusions

The findings from this thesis help in the development of farm biosecurity guidelines and risk reduction measures suitable for the local context. An education campaign for rural pig farmers in Thailand is urgently required in order to successfully prevent the interspecies transmission and control of influenza A virus on pig farms. Virological surveillance in Thai pigs from commercial farms should be continued to monitor genetic characteristics and to explore the evolutionary diversity of influenza A viruses. In Cambodia, it is imperative to implement a well-designed surveillance system for influenza A in pigs. In the context of limited resources, sero-surveillance for influenza A viruses in pigs in high-risk areas or at slaughterhouses is recommended.
Appendix 1

Questionnaire for a study on potential risks of influenza A virus transmission on farms in rural Thailand

| Name of farmer: ____________________ | Telephone No: ____________________ |
| Address: ________________________________________________________________ |
| Name of Interviewer: ____________________ | Telephone No: ____________________ |

PART A: FARMER’S EDUCATIONAL BACKGROUND

A1. What is your education background?
   - (1) Completed primary school
   - (2) Completed secondary school
   - (3) Completed high school
   - (4) Completed bachelor’s degree
   - (5) Other ____________________

A2. Is raising pigs your main occupation? And if NO, what is your main occupation?
   - (1) Yes - Pig farmer
   - (2) No - Government officer/Soldier/Police
   - (3) No – Crop farmer
   - (4) No - Other__________________

A3. Why do you raise pigs? (Tick more than 1 box if necessary)
   - (1) Earn money
   - (2) Own consumption
   - (3) Family business
   - (4) Other__________________

A4. How long have you been a pig farmer?
PART B: FARM STRUCTURE AND HERD INFORMATION

B1. What are your purposes for pig farming? (Tick more than 1 box if necessary)

- (1) Selling weaning pigs
- (2) Selling fattening pigs
- (3) Selling breeders
- (4) Breeding services

B2. What type of animals and how many do you have on your farm?

1. Pigs________
2. Chickens________
3. Cattle________
4. Dogs________
5. Cats________
6. Other __________

B3. Which of the following animals live or come within a 2.5 m radius of your pigs?

1. Chickens________
2. Cattle________
3. Dogs________
4. Cats________
5. Other __________

B4. Do you have your own boar? If yes, why do you have a boar?

- (1) No – Hire boars from outside
- (2) Yes – For own breeding
- (3) Yes – For own breeding and hiring out to others
- (4) Yes – For hiring out to others
PART C: HUSBANDRY

<table>
<thead>
<tr>
<th>C1. Can your piglets run outside their pens?</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ (1) Yes</td>
</tr>
<tr>
<td>□ (2) No</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C2. Can pigs from outside your herd have direct contact with your pigs? If they can make contact, please specify where they come from?</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ (1) No contact</td>
</tr>
<tr>
<td>□ (2) From neighbouring area</td>
</tr>
<tr>
<td>□ (3) Within a village</td>
</tr>
<tr>
<td>□ (4) Other ________________________________________________________</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C3. What materials are mostly used to construct the pens?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Roof</td>
</tr>
<tr>
<td>□ (1) No roof</td>
</tr>
<tr>
<td>□ (2) Leaves</td>
</tr>
<tr>
<td>□ (3) Tile</td>
</tr>
<tr>
<td>□ (4) Metal sheet</td>
</tr>
<tr>
<td>2) Wall</td>
</tr>
<tr>
<td>□ (1) No wall</td>
</tr>
<tr>
<td>□ (2) Wood</td>
</tr>
<tr>
<td>□ (3) Cement</td>
</tr>
<tr>
<td>□ (4) Other ________</td>
</tr>
<tr>
<td>3) Floor</td>
</tr>
<tr>
<td>□ (1) Ground</td>
</tr>
<tr>
<td>□ (2) Pit</td>
</tr>
<tr>
<td>□ (3) Cement</td>
</tr>
<tr>
<td>□ (4) Other ________</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C4. Can excessive water from rain enter your pig pens?</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ (1) Yes</td>
</tr>
<tr>
<td>□ (2) No</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C5. Do you usually clean the pens? If yes, how do you clean the pens?</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ (1) Sweep</td>
</tr>
<tr>
<td>□ (2) Rinse with water</td>
</tr>
<tr>
<td>□ (3) Apply a disinfectant</td>
</tr>
<tr>
<td>□ (4) Other ________________</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>C6. What do you do with the manure from your pigs?</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ (1) Do Nothing</td>
</tr>
<tr>
<td>□ (2) Use on garden</td>
</tr>
<tr>
<td>□ (3) Give to neighbours</td>
</tr>
<tr>
<td>□ (4) Sell to buyers</td>
</tr>
</tbody>
</table>
C7. Do you wear any protective clothing/gear when entering the pig pens? If yes – What protective clothing do you wear? (Tick more than one box if necessary)

- (1) Boots
- (2) Gloves
- (3) Mask
- (4) None

C8. Do you require visitors from outside to have clean hands or shoes before entering your pig pens?

- (1) Yes
- (2) No

C9. What kind of food do you feed your pigs?

- (1) Swill – from own kitchen
- (2) Vegetables
- (3) Bran
- (4) Manufactured food
- (5) Other _______________

C10. How long do you work with your pigs each day? __________ minutes/ day

C11. Approximately how many times do you touch your pigs each day?

- (1) Never
- (2) Less than once per day
- (3) Once per day
- (4) More than once per day

C12. Can vehicles enter your farm and go within 2.5m of the pig pens?

- (1) Yes
- (2) No

C13. What is the water source for your pigs?

- (1) Household well
- (2) Village well
<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(3) Rain</td>
<td>(4) Canal/river</td>
<td>(5) Tap</td>
<td>(6) Other _________</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14. What identification method do you use for your sows and boars?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) None</td>
<td>(2) Remember their attributes</td>
<td>(3) Tattoo</td>
<td>(4) Ear tag</td>
<td>(5) Ear mark</td>
<td>(6) Other ____________</td>
<td></td>
</tr>
<tr>
<td>C15. What identification method do you use for your piglets?</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) None</td>
<td>(2) Remember their attributes</td>
<td>(3) Tattoo</td>
<td>(4) Ear tag</td>
<td>(5) Ear mark</td>
<td>(6) Other ____________</td>
<td></td>
</tr>
</tbody>
</table>

**PART D: PIG MOVEMENT AND TRADING**

D1. Have you sold any pigs in the last 12 months?
   □ (1) Yes
   □ (2) No

D2. If so – where and who did you sell to?
   □ (1) At the market
   □ (2) Middle man come to buy at home
   □ (3) Neighbours sell them
   □ (4) Other ____________

D3. Please specify the month you are most likely to sell your pig(s)?
Why do you sell pigs in this month ____________________________

D4. Please specify details of each sale during the last 12 months

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Price/head</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PART E: PIGS’ HEALTH STATUS AND VACCINATION

E1. In the last 12 months did you have any sick pigs? If yes what were the signs of illness you saw?

- □ (1) No sick pigs
- □ (2) Yes - No clinical signs seen
- □ (3) Yes - Sudden death
- □ (4) Yes - Nasal discharge
- □ (5) Yes - Coughing/Sneezing
- □ (6) Yes - Abortion
- □ (7) Yes - Skin disease
- □ (8) Yes - High fever
- □ (9) Yes - Inappetite
- □ (10) Yes - Diarrhoea
- □ (11) Yes - Vomiting
- □ (12) Yes - Joint swelling
- □ (13) Yes - Other

E2. Generally, what do you do when you have sick pigs? (Tick more than one option if necessary)

- □ (1) Do nothing
- □ (2) Isolate sick pigs from others
- □ (3) Treat sick pigs by yourself
- □ (4) Sell sick pigs
- □ (5) Slaughter for consumption
- □ (6) Slaughter for selling
- □ (7) Contact someone to get help (go to E3)

E3. Who, if anyone, would you contact if you had sick pigs?

- □ (1) No one
- □ (2) Paravets
E4. Please specify the months you are most likely to see sick pigs? __________

E5. Please specify the months you are least likely to see sick pigs? __________

E6. When a pig dies – What do you usually do with the body of the dead pig?
- (1) Burn
- (2) Bury
- (3) Eat
- (4) Other

E7. Have your pigs been vaccinated against any diseases in the last 12 months? If yes what diseases have they been vaccinated against? (Tick more than one box if necessary)
- (1) None
- (2) FMD
- (3) CSF
- (4) Pseudorabies
- (5) Other _________

E8. If your neighbours have pigs, have you heard if any of them have had pigs sick with respiratory signs in the past 12 months?
- (1) Yes
- (2) No

PART F: FARMERS’ HEALTH STATUS

F1. Do you wash hands after touching pigs?
- (1) Yes-all the time
- (2) Yes-most times
- (3) Yes-some of the time
- (4) Yes-rarely
- (5) No-never
<table>
<thead>
<tr>
<th></th>
<th>F2. How do you usually wash your hands?</th>
</tr>
</thead>
<tbody>
<tr>
<td>□</td>
<td>(1) With water</td>
</tr>
<tr>
<td>□</td>
<td>(2) With water and soap</td>
</tr>
<tr>
<td>□</td>
<td>(3) With an antibacterial solution</td>
</tr>
<tr>
<td>□</td>
<td>(4) With other ________________________</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>F3. In the past 12 months have you or anyone in your household been sick? And if YES what were the clinical signs? (Tick more than one box if necessary)</th>
</tr>
</thead>
<tbody>
<tr>
<td>□</td>
<td>(1) No</td>
</tr>
<tr>
<td>□</td>
<td>(2) Yes - Nasal discharge</td>
</tr>
<tr>
<td>□</td>
<td>(3) Yes - Coughing/Sneezing</td>
</tr>
<tr>
<td>□</td>
<td>(4) Yes - Sore throat</td>
</tr>
<tr>
<td>□</td>
<td>(5) Yes - High fever</td>
</tr>
<tr>
<td>□</td>
<td>(6) Yes - Other (specify) ___________</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>F4. Did the sick person come within 2.5 m of your pigs?</th>
</tr>
</thead>
<tbody>
<tr>
<td>□</td>
<td>(1) Yes</td>
</tr>
<tr>
<td>□</td>
<td>(2) No</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>F5. Do you know the cause of the sickness?</th>
</tr>
</thead>
<tbody>
<tr>
<td>□</td>
<td>(1) Yes please specify__________________</td>
</tr>
<tr>
<td>□</td>
<td>(2) No</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>F6. Have you ever been vaccinated against the influenza A(H1N1)pdm09?</th>
</tr>
</thead>
<tbody>
<tr>
<td>□</td>
<td>(1) Yes - please specify <strong>/</strong>/__</td>
</tr>
<tr>
<td>□</td>
<td>(2) No</td>
</tr>
</tbody>
</table>

**THANK YOU FOR YOUR PARTICIPATION**
Appendix 2

Questionnaire for a study on pig marketing among traders in rural Thailand

Name of farmer: _______________  Telephone No: _______________
Address_____________________________________________________________
Name of Interviewer: _______________  Telephone No: _______________

1. What kind of pigs have you sold during the last year? How many did you sell and what price did you obtain per head?

<table>
<thead>
<tr>
<th>(1) Live pigs</th>
<th>□ Sow</th>
<th>□ Boar</th>
<th>□ Small pig (0-45 days old)</th>
<th>□ Medium pig (3-6 months old)</th>
<th>□ Large pig (&gt;6 months old)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Month</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of pigs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Price/head</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(2) Carcasses</th>
<th>□ Sow</th>
<th>□ Boar</th>
<th>□ Small pig (0-45 days old)</th>
<th>□ Medium pig (3-6 months old)</th>
<th>□ Large pig (&gt;6 months old)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Month</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of pigs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Price/head</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. In the last year where did you get new pigs from?

□ (1) Own farms

□ (2) Farmers - please specify village & district name________________

□ (3) Middle men/distributors - please specify village & district name__________

□ (4) Middle men from Laos

□ (5) Other: please specify________________

3. Do you usually transport pigs to the market/ selling place by yourself?

□ (1) Yes - By myself
<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>□ (2) No - By pig farmers</td>
<td></td>
</tr>
<tr>
<td>□ (3) No - By hired workers</td>
<td></td>
</tr>
<tr>
<td>□ (4) No – Other ____________</td>
<td></td>
</tr>
</tbody>
</table>

4. If you buy pigs from farmers, how many farms do you visit per trading? ___________ farms

5. Do you have your own vehicle?
   □ (1) Yes - please specify type of vehicle_________
   □ (2) No - Go to question no. 8

6. Do you clean your vehicle? If yes, how do you clean and how often?
   □ (1) Yes - please specify cleaning method_________
   & please specify frequency_________
   □ (2) No

7. Do you disinfect your vehicle? If yes, how do you disinfect and how often?
   □ (1) Yes - please specify disinfectant_________
   & please specify frequency_________
   □ (2) No

8. Do you know if you need any specific document to transport pigs?
   □ (1) Yes - please specify type of document_________
   □ (2) No

9. If you sell carcasses, where are the pigs slaughtered?
   □ (1) On farm
   □ (2) At the slaughterhouse - please specify the location__________________
   □ (3) At the restaurant - please specify the location__________________
   □ (4) Other (please specify)__________________

10. Who are your customers?
    □ (1) villagers - please specify village & district name______________
11. How often do you sell pigs in this market/selling place? And how many?

<table>
<thead>
<tr>
<th>Frequency</th>
<th>No. of pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ (1) Everyday</td>
<td></td>
</tr>
<tr>
<td>□ (2) Every week</td>
<td></td>
</tr>
<tr>
<td>□ (3) Every month</td>
<td></td>
</tr>
<tr>
<td>□ (4) Irregular</td>
<td></td>
</tr>
<tr>
<td>depends on _________</td>
<td></td>
</tr>
<tr>
<td>□ (5) Other (please specify)</td>
<td>_________</td>
</tr>
</tbody>
</table>

12. In the last year did you have sick pigs?

□ (1) Yes - please specify clinical signs_________
□ (2) No

13. What would you do if you found sick pigs?

□ (1) Buy cheap and sell out early
□ (2) Buy cheap and put on quarantine
□ (3) Don’t buy and report to authorities
□ (4) Don’t buy and do nothing

14. In last year have you reported any sick pigs to the authorities? If no, why?

□ (1) Yes - Go to question no. 15
□ (2) No, I do not know how to.
□ (3) No, I believe I would have a problem selling the pigs if the sickness was reported.
□ (4) No, I am scared my pigs would be culled.
□ (5) Other: please specify ____________________
<table>
<thead>
<tr>
<th>15. Who did you report to?</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ (1) Village chiefs</td>
</tr>
<tr>
<td>□ (2) Paravets</td>
</tr>
<tr>
<td>□ (3) District veterinary officers</td>
</tr>
<tr>
<td>□ (4) Other please specify_______________</td>
</tr>
</tbody>
</table>

THANK YOU FOR YOUR PARTICIPATION
Appendix 3

Questionnaire for a knowledge, attitudes and practices (KAPs) survey towards swine influenza amongst pig farmers in Mukdahan

Name of farmer: ______________________ Telephone No: ______________
Address_____________________________________________________________________
Name of Interviewer: ______________ Telephone No: ______________

I. BACKGROUND

<table>
<thead>
<tr>
<th>1. Age</th>
<th>Years</th>
</tr>
</thead>
</table>
| 2. Gender | ☐ (1) Male  
☐ (2) Female |
| 3. Marital status | ☐ (1) Married  
☐ (2) Single  
☐ (3) Other: please specify__________________ |
| 4. Number of children | ☐ (1) No formal education  
☐ (2) Primary school  
☐ (3) Secondary school  
☐ (4) High school  
☐ (5) Technical college  
☐ (6) University  
☐ (7) Other: please specify__________________ |
| 5. What is your highest education level? | ☐ (1) No formal education  
☐ (2) Primary school  
☐ (3) Secondary school  
☐ (4) High school  
☐ (5) Technical college  
☐ (6) University  
☐ (7) Other: please specify__________________ |
| 6. Years earning a living as a farmer | years |
| 7. Number of pigs in farm operation | |

II. BASIC KNOWLEDGE

| 1. Have you heard of swine influenza? | ☐ (1) Yes  
☐ (2) No  
☐ (3) Not sure |
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2. What is swine influenza?</td>
<td></td>
</tr>
<tr>
<td>3. What signs would you associate with swine influenza in pigs?</td>
<td></td>
</tr>
<tr>
<td>4. What are the main modes of transmission of swine influenza</td>
<td></td>
</tr>
</tbody>
</table>
### III. DISEASE RISK PERCEPTION AND PREVENTION ON SWINE INFLUENZA

1. What factors do you think pose a risk of your pigs getting influenza? (1=Unable to Judge, 2=No risk, 3=Low risk, 4=Medium risk, 5=High risk)

<table>
<thead>
<tr>
<th>Measurement</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Introduction of new pigs</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>(2) Neighbours’ pigs close to your pigs</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>(3) People, equipment and vehicles entering farms</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>(4) Many kinds of animals raised on farm</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>(5) Pigs with flu-like symptoms</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>(6) People with flu-like symptoms</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

2. What hygienic practices do you adopt to avoid spreading of swine influenza viruses?

- □ (1) None
- □ (2) Only touch pigs if I am wearing gloves
- □ (3) Change clothes after touching pigs or their faeces
- □ (4) Wash hands with soap after handling pigs or manure
- □ (5) Wear face masks while working in farm

3. How do you ensure only healthy pigs are purchased?

- □ (1) Unsure of what to do
- □ (2) Only concerned with the price
- □ (3) Observe physical condition
- □ (4) I know the sellers and trust them
- □ (5) Quarantine for ____ days before introducing new pigs into a herd
- □ (6) Other: please specify __________

4. Please explain what you would do if you suspected your pigs had swine influenza?

- □ (1) Treat myself please specify type of medications used________
- □ (2) Eat sick pigs
- □ (3) Give away to friends
- □ (4) Sell pigs to neighbours
- □ (5) Report suspect cases to authority
- □ (6) Do nothing
- □ (7) Other: please specify __________
IV. FARMERS’ ATTITUDES AND BEHAVIOUR REGARDING REPORTING AND DISEASE CONTROL

| 1. If you detected sick pigs with respiratory signs, would you report the pig sickness to the authorities? | □ (1) Yes, I would.  
□ (2) No, I do not know how to.  
□ (3) No, I believe I would have a problem selling the pigs if the sickness was reported.  
□ (4) No, I am scared my pigs would be culled. |
|---|---|
| 2. Do you believe that it is important to report any pig sickness or deaths, why? | □ (1) Yes - The sickness or death may be due to infectious diseases.  
□ (2) Yes - The pig owners may receive management advice from authorities.  
□ (3) No – I believe the sickness or death may not cause a problem.  
□ (4) No - No help would be provided from veterinary staff or authorities. |
| 3. What do you see as necessary to prevent or control swine influenza? (1= Strongly disagree, 2= Disagree, 3=Somewhat disagree, 4= Unsure, 5= Somewhat agree, 6= Agree 7= Strongly agree) | Measurement |
| (1) Early disease detection of swine influenza in pigs | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| (2) More education and awareness on prevention of swine influenza | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| (3) Authorities to advise me when pigs are sick with respiratory signs | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| (4) Regular veterinary visits | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| (5) Safe source of pigs | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| (6) Control pig movement from outbreak areas of swine influenza | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| (7) Prevent contact between pigs on farm and neighboring farms | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| (8) Vaccination of swine influenza in pigs | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| (9) Vaccination of seasonal influenza | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
in humans

4. What level of importance do you consider the following factors in your decision to report pigs with unusual symptoms? (0=No Importance, 1=very low importance, 2=low importance, 3=medium importance, 4=high importance, 5=very high importance)

<table>
<thead>
<tr>
<th>Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Having a good connection with authorities</td>
</tr>
<tr>
<td>0   1   2   3   4   5</td>
</tr>
<tr>
<td>(2) Having easy access to authorities</td>
</tr>
<tr>
<td>0   1   2   3   4   5</td>
</tr>
<tr>
<td>(3) The government making good advertising campaign to get farmers’ cooperation</td>
</tr>
<tr>
<td>0   1   2   3   4   5</td>
</tr>
<tr>
<td>(4) Practical control measures according to DLD regulations</td>
</tr>
<tr>
<td>0   1   2   3   4   5</td>
</tr>
<tr>
<td>(5) Provision of compensation for the cost of control</td>
</tr>
<tr>
<td>0   1   2   3   4   5</td>
</tr>
</tbody>
</table>

5. If you had any sick pigs/dead pigs, how likely is it that you would report to ________? (Negligible = 0; Very low = 1; Low = 2; Moderate =3; High = 4; Very high = 5)

<table>
<thead>
<tr>
<th>Measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Village chiefs</td>
</tr>
<tr>
<td>0   1   2   3   4   5</td>
</tr>
<tr>
<td>(2) Paravets</td>
</tr>
<tr>
<td>0   1   2   3   4   5</td>
</tr>
<tr>
<td>(3) District veterinary officers</td>
</tr>
<tr>
<td>0   1   2   3   4   5</td>
</tr>
<tr>
<td>(4) Other please specify___________</td>
</tr>
<tr>
<td>0   1   2   3   4   5</td>
</tr>
</tbody>
</table>

V. PERSONAL HABITS AND PRACTICES

1. How do you usually wash your hands?
   □ (1) With water
   □ (2) With water and soap
   □ (3) With an antibacterial solution

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2. Do you wash hands after touching pigs?  
☐ (4) With other ________________  
☐ (1) Yes-all the time  
☐ (2) Yes-most times  
☐ (3) Yes-some of the time  
☐ (4) Yes-rarely  
☐ (5) No-never  
☐ (6) Not sure

3. Do you touch sick or dead pigs with bare hands?  
☐ (1) Yes-all the time  
☐ (2) Yes-most times  
☐ (3) Yes-some of the time  
☐ (4) Yes-rarely  
☐ (5) No-never  
☐ (6) Not sure

4. Will you work if you have flu-like symptoms?  
☐ (1) Yes-all the time  
☐ (2) Yes-most times  
☐ (3) Yes-some of the time  
☐ (4) Yes-rarely  
☐ (5) No-never  
☐ (6) Not sure

5. Do you avoid contact with people who have symptoms of flu?  
☐ (1) Yes-all the time  
☐ (2) Yes-most times  
☐ (3) Yes-some of the time  
☐ (4) Yes-rarely  
☐ (5) No-never  
☐ (6) Not sure

VI. DISEASE AWARENESS

1. Where did you learn about swine influenza?  
☐ (1) Village livestock workers  
☐ (2) Veterinarians  
☐ (3) Village chief or community leaders  
☐ (4) Neighbours  
☐ (5) Friends  
☐ (6) Family  
☐ (7) Radio & Television  
☐ (8) Newspapers  
☐ (9) Pamphlets/brochure/poster  
☐ (10) Other: Please specify__________

2. Are you interested in receiving further information on swine influenza?  
☐ (1) Yes  
☐ (2) No  
☐ (3) Not sure

3. What special information on swine influenza would you like to know?  
☐ (1) Basic knowledge  
☐ (2) Prevention methods  
☐ (3) Treatment
### DISEASE RISK PERCEPTION ON INFLUENZA A(H1N1)pdm09

<table>
<thead>
<tr>
<th>1. Have you heard of influenza A(H1N1)pdm09?</th>
<th>□ (1) Yes □ (2) No-Go to question no.5 □ (3) Not sure-Go to question no.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Where did you learn about influenza A(H1N1)pdm09?</td>
<td>□ (1) Village livestock workers □ (2) Veterinarians □ (3) Village chief or community leaders □ (4) Neighbours □ (5) Friends □ (6) Family □ (7) Radio &amp; Television □ (8) Newspapers □ (9) Pamphlets/brochure/poster □ (10) Other: Please specify__________________</td>
</tr>
<tr>
<td>3. Do you have concerns that you might get sick from A(H1N1)pdm09 virus?</td>
<td>□ (1) no concern □ (2) very low concern □ (3) low concern □ (4) medium concern □ (5) high concern □ (6) very high concern</td>
</tr>
<tr>
<td>4. Have you taken special precautions to protect yourself against A(H1N1)pdm09 virus?</td>
<td>□ (1) none □ (2) increased frequency of hand washing □ (3) bought anti-viral medication □ (4) bought any type of face mask □ (5) avoided public gatherings and contact with others □ (6) avoided travel □ (7) stockpiled food/water</td>
</tr>
</tbody>
</table>
5. Have you ever had seasonal influenza vaccination? If not, why?
   □ (1) Yes-Go to question no.6
   □ (2) No-Go to question no.7

6. Why have you had seasonal influenza vaccination?
   □ (1) I’m scared I would get a flu.
   □ (2) The government had a campaign for free seasonal influenza vaccine
   □ (3) I think the cost of vaccination is not expensive
   □ (4) Other (please specify)________________

7. Why haven’t you had seasonal influenza vaccination?
   □ (1) I think influenza is not an important disease.
   □ (2) I think the cost of vaccination is expensive.
   □ (3) I think the medical centre is too far.
   □ (4) Other (please specify)________________

THANK YOU FOR YOUR PARTICIPATION
REFERENCES


antigenically distinguishable from classical and European strains. *The Veterinary Record* 132:598-602.


Pensaert, M., K. Ottis, J. Vandeputte, M.M. Kaplan, and P.A. Bachmann. 1981. Evidence for the natural transmission of influenza A virus from wild ducks to


