

Zoonoses in Rural Cambodia

A One Health Perspective on Influenza and *Campylobacter*

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Abstract

Zoonotic diseases, transmissible between animals and humans, make up the majority of emerging infectious diseases, posing a threat to public health and global food security. The emergence of infections is partly driven by close contact between humans and livestock, which is common in smallholder livestock farming in rural tropical areas. The aim of this thesis was to provide information on the animal-human interplay in rural tropical areas in general and in Cambodia in particular, focusing on influenza A virus and *Campylobacter* as examples of zoonotic pathogens.

Interviews were carried out in 300 rural households and samples were collected in the same households from humans and livestock, primarily chickens, ducks, pigs and cattle. In the households studied, a clear gender division in livestock responsibility was observed. Practices associated with zoonosis exposure were common, but the threat of zoonoses was not reported to be a concern. Furthermore, knowledge and awareness of zoonoses did not markedly reduce practices associated with increased zoonosis exposure, thereby revealing a knowledge-to-behaviour gap.

Sampled pigs and poultry had 1.3% overall prevalence of influenza A virus. Highly pathogenic subtypes were not found, but virus reassortment, involving potentially zoonotic and pandemic subtypes, seemed to occur frequently. Routine culture was insufficiently sensitive in detecting *Campylobacter* in field samples frozen before analysis. In contrast, PCR proved more sensitive and *C. jejuni*, *C. coli* or both were detected in 8% of adults, 19% of children, 56% of chickens, 24% of ducks, 72% of pigs and 5% of cattle. Moreover, a number of household practices along the meat production chain, from livestock rearing and slaughter to meat consumption, were associated with human *C. jejuni* and *C. coli* positivity.

In conclusion, presence of pathogens with zoonotic potential and insufficient zoonosis management was shown on Cambodian smallholdings. The novel data presented on zoonosis epidemiology and household risk factors can help guide future interventions in zoonosis prevention, detection and control for improved health and livelihoods in rural tropical areas.

Keywords: *Campylobacter coli*, *Campylobacter jejuni*, Household practice, Human, Influenza A virus, Livestock, Risk factor, Rural household, Zoonosis.

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Dedication

To my family

Overcoming poverty is not a task of charity, it is an act of justice.

Nelson Mandela

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Osbjer K, Boqvist S, Sokerya S, Kannarath C, San S, Davun H and Magnusson U (2015). Household practices related to disease transmission between animals and humans in rural Cambodia. *BMC Public Health*, 15 (476).
- II Osbjer K, Sokerya S, San S, Davun H, Chheng K, Magnusson U, Olsen B, Berg M, Zohari S (2016). Influenza A in backyard pigs and poultry in rural Cambodia. *Submitted manuscript*.
- III Osbjer K, Tano E, ChhayHeng L, Mac-Kwashie AO, Fernström L.-L, Ellström P, Sokerya S, Sokheng C, Mom V, Chheng K, San S, Davun H, Boqvist S, Rautelin H, Magnusson U (2016). Detection of *Campylobacter* in human and animal field samples in Cambodia. *Apmis*, 124(6), 508-515.
- IV Osbjer K, Boqvist S, Sokerya S, Chheng K, San S, Davun H, Rautelin H, Magnusson U (2016). Risk factors associated with *Campylobacter* detected by PCR in humans and animals in rural Cambodia. *Submitted manuscript*.

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Abbreviations

AIV	Avian Influenza Virus
<i>C. coli</i>	<i>Campylobacter coli</i>
<i>C. jejuni</i>	<i>Campylobacter jejuni</i>
CI	Confidence Interval
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization of the UN
FTA	Flinders Technology Associates
GEE	Generalised estimating equations
HP	Highly Pathogenic
HPAI	Highly Pathogenic Avian Influenza
IAV	Influenza A Virus
ICC	Intra-cluster correlation coefficient
LPAI	Low Pathogenic Avian Influenza
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MDG	The UN Millennium Development Goal
NaVRI	National Veterinary Research Institute, Cambodia
NIPH	National Institute of Public Health, Cambodia
OIE	World Organization for Animal Health
OR	Odds ratio
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRT-PCR	Real-time reverse transcription polymerase chain reaction
RT-PCR	Real-time polymerase chain reaction
SEP	Socio-economic position
SIV	Swine Influenza Virus
SLU	Swedish University of Agricultural Sciences

SVA	National Veterinary Institute, Sweden
TR	Triple reassortant
UU	Uppsala University, Sweden
WHO	World Health Organization

1 Introduction

Zoonoses, diseases transmissible between animals and humans, cause the majority of human infectious diseases worldwide and have been responsible for serious disease outbreaks in recent years. However, despite a huge global impact on human and animal health, there are still gaps in the overall understanding of how zoonoses spread, develop and emerge.

1.1 Livestock for food security and livelihoods

In a global population of 7.4 billion, 793 million are estimated to be suffering from hunger (FAO, 2016) and 896 million to be living in poverty, on less than USD 1.90 a day (WB, 2012). To achieve global economic, social and environmental sustainable development and end all forms of poverty and hunger, in September 2015 the United Nations adopted the 2030 Sustainable Development Agenda as the successor to the Millennium Development Goals (MDGs). The agenda, focusing on different aspects of sustainable poverty reduction, was defined in 17 goals, with goal number two targeting achievement of food security, improved nutrition and sustainable agriculture (UN, 2015).

Agriculture engages many of the world's poorest. Around one billion poor people are estimated to derive at least part of their living from livestock, making livestock the most important sector for the lives and livelihoods of the global poor (Figure 1) (Steinfeld *et al.*, 2006). In addition to food, livestock provide important products and services such as social status, asset savings, draught power, fibres, and manure for fuel and fertilisers. Furthermore, the livestock sector performs important development functions, with contributions to nutritious diets, economic growth and livelihoods (FAO, 2016). The expected global population growth, increased income and expanding urbanisation are predicted to increase the global demand for livestock products

by 70% in the coming 25 years, making livestock even more important in the future (FAO, 2016; Steinfeld *et al.*, 2006).

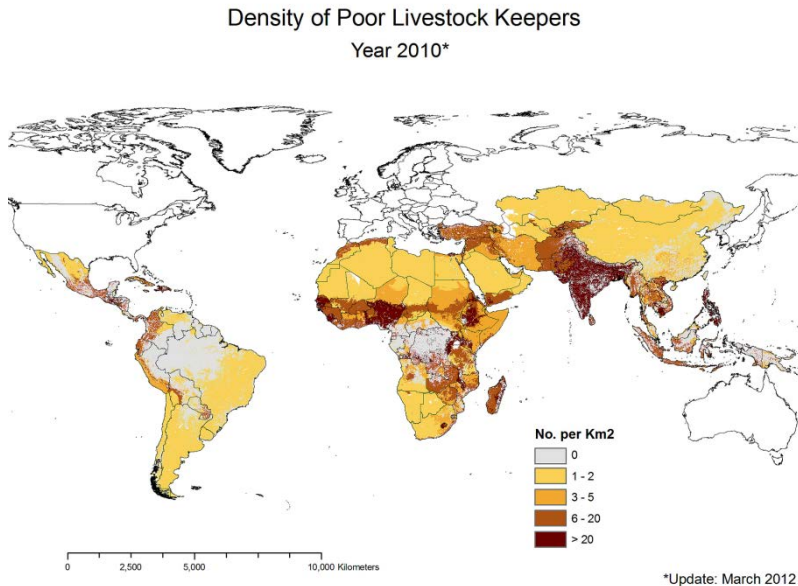


Figure 1. The global density of poor livestock keepers (Grace *et al.*, 2012).

1.2 Livestock and zoonoses in low-income countries

While keeping livestock and having access to animal-based food is essential for enhanced global food and nutrition security, certain types of livestock systems are associated with environmental degradation, greenhouse gas emissions and zoonotic and food-borne diseases (Kristjanson *et al.*, 2014; Randolph *et al.*, 2007). Zoonoses are pathogens transmissible between animals and humans. They may be spread through direct contact, indirect contact (*via* food/water or an environment reservoir) or *via* vectors (biting or mechanical transfer by insects) (Taylor *et al.*, 2001). Zoonotic pathogens can be divided into viruses, prions, bacteria, rickettsia, fungi, protozoa and helminths (Taylor *et al.*, 2001) and their occurrence is endemic/enzootic (constantly maintained in the population at the frequency expected), epizootic (occurrence at a higher frequency than expected, analogous to an epidemic in humans) or emerging (recently appeared in a population or known for some time, but rapidly increasing in incidence or geographical range) (Grace *et al.*, 2012). More than 60% of human diseases that have emerged during the past half a century are zoonoses (Jones *et al.*, 2008) and endemic/enzootic zoonoses cause about a

billion cases of illness in people worldwide and millions of deaths every year (Karesh *et al.*, 2012).

Associations between poverty, livestock keeping and zoonoses have been proposed (Grace *et al.*, 2012). High livestock density, mainly determined by high human density, drives the transmission of zoonoses through increased probability and frequency of contacts (Grace *et al.*, 2012). In low-income countries, close contact between humans and livestock is commonly enabled by free-ranging livestock and livestock pens bordering the house. This close proximity, together with unsanitary conditions and limited access to health and veterinary services, make the burden of zoonotic diseases disproportionately high in poor communities (Randolph *et al.*, 2007). For human diseases, the burden can be measured by non-financial methods, often referred to as health-adjusted life years (HALYs), with disability-adjusted life years (DALYs) being the most well-known (Carabin *et al.*, 2005). DALYs are quantified as the present value of future years lost due to premature death or living with poor health (Murray *et al.*, 2000). However, being a human health measure, it only captures some of the burdens imposed by zoonoses.

1.3 Cambodia - livestock and health

Cambodia is located in Southeast Asia, bordered by Thailand, Lao PDR, Vietnam and the Gulf of Thailand. The country covers 181,035 square kilometres and consists of 27 provinces. Cambodia is a tropical country with marked dry and rainy seasons, with the dry season generally running from November to April and the rainy season from May-June to October-November (Mardy *et al.*, 2009). Further facts on Cambodia can be found in Figure 2.

Despite strong annual growth in gross domestic product (GDP), a large flow of foreign funding and progress in reducing poverty, Cambodia remains among the poorest countries in Southeast Asia, with uneven urban-rural income (WB, 2015; NIS, 2013; Engvall *et al.*, 2008). The labour force is overwhelmingly agriculture-orientated and almost 90% of the Cambodian poor live in the countryside and lack adequate healthcare, access to improved sanitation, proper education and social welfare (WB, 2016; Burgos *et al.*, 2008). The under-five mortality rate is high, with diarrhoea accounting for 14% of deaths. Malnutrition is widespread, *e.g.* as an average for all of Cambodia, in 2012 28% of children under five were estimated to be underweight and 40% to be stunted (below the median height-for-age) (WHO, 2012). A protective factor in malnutrition, especially in resource-poor rural households, is consumption of animal-based food (Darapheak *et al.*, 2013). Livestock thus have an important function by contributing to better nutrition and food security.

Cambodia country facts


Official name:	Kingdom of Cambodia	
Capital City:	Phnom Penh	
Population:	15.7 million	
Population Density:	87 per square km	
Rural Population:	79.3%	
Life Expectancy:	64.1 years	
Language (official):	Khmer	
Agriculture - GDP:	28.6%	
Industry - GDP:	27.9%	
Service - GDP:	43.6%	
Government:	Multiparty Democracy, Constitutional Monarchy	
Religion (official):	Buddhist (96.9% of the population)	
Human Development Index :	0.555 (ranking 143 out of 188 countries in the 2014 index)	
Gender Inequality Index:	0.477 (ranking 104 out of 155 countries in the 2014 index)	

Figure 2. Cambodia country facts (CIA, 2016; WB, 2016; UNDP, 2012).

Livestock are deeply embedded in society and customs in Cambodia and are an integral feature of smallholder agriculture. A large number of rural households keep multi-purpose livestock, mainly birds, pigs, cattle and/or water buffalo to meet household consumption needs, social obligations and minor cash expenses (Pfeiffer *et al.*, 2013). Although most livestock producers are smallholders with small-scale, extensive backyard/garden production, a diverse range of production forms co-exist, including semi-intensive, small to medium scale, market-orientated commercial production and intensive, large-scale, industrially integrated production (Burgos *et al.*, 2008).

Regardless of farm size and the species reared, livestock diseases, when they occur, cause severe loss of income due to morbidity and mortality, as well as lost opportunity costs in terms of livestock sector development. Major disease outbreaks normally affect both infected and non-infected farms, owing to the decreased price of meat and animal products as consumer demand for those products declines (Basuno *et al.*, 2010). In early 2016, Cambodia's first veterinary legal framework was endorsed¹. The law includes regulations on

1. Sothyra Tum, Director of National Veterinary Research Institute, personal communication 2016-03-09

disease control, but the national diversity in livestock production and limited institutional coordination make national policies for livestock health and production challenging to implement effectively (Burgos *et al.*, 2008).

In 2014, the Department of Animal Health and Production in Cambodia estimated the livestock population to be: 17.8 million chickens, 7.6 million ducks, 2.4 million pigs, 3.1 million cattle and 542 000 buffalo (MAFF, 2014). Maps of Cambodia with the poultry, pig, cattle and buffalo density can be found in Figure 3.

1.3.1 Zoonoses in Cambodia and Southeast Asia

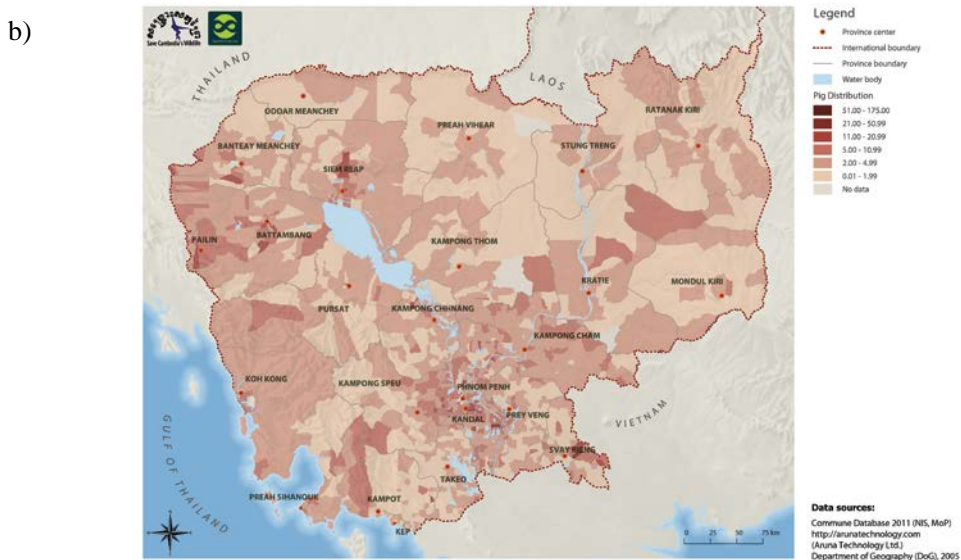
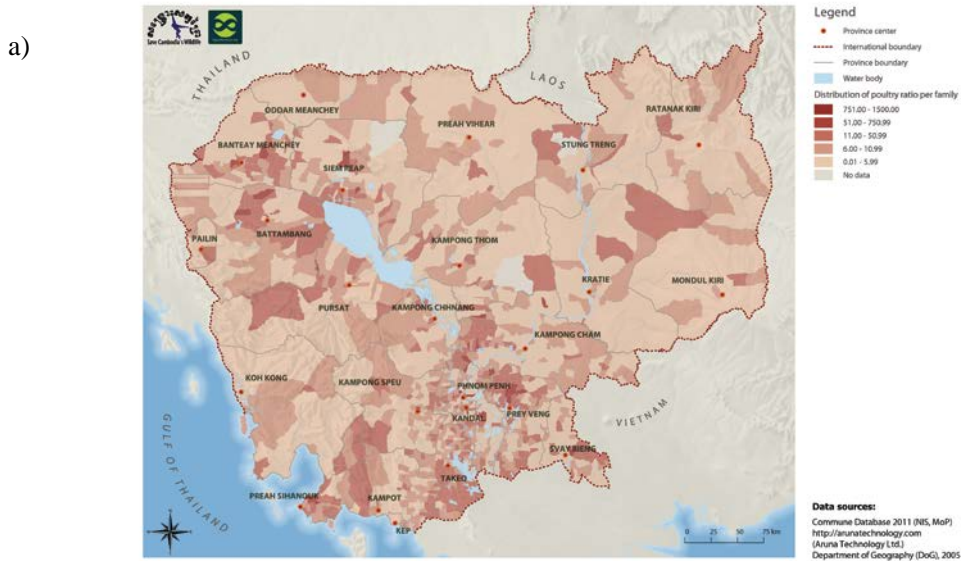
Southeast Asia has been identified as a potential global hotspot for emerging infectious diseases, in particular zoonotic diseases (Coker *et al.*, 2011; Jones *et al.*, 2008). The potential for disease emergence in the region is enabled by the complex interactions between humans, livestock, wildlife and agricultural land, which allow microbes to exploit new ecological niches (Wei *et al.*, 2015). Regional population growth, mobility, urbanisation, climate change and environmental changes, such as livestock intensification and deforestation, are driving the processes further (Coker *et al.*, 2011; Jones *et al.*, 2008).

A number of recent zoonotic epidemics have struck Southeast Asia during the past two decades, including Severe Acute Respiratory Syndrome (SARS) and Highly Pathogenic (HP) Avian Influenza H5N1 (Coker *et al.*, 2011). In Cambodia, HP H5N1 has caused numerous poultry outbreaks and a few hundred human cases which have contributed to better collaboration between the human and animal health sectors. However, the response to avian influenza has also generated criticism due to the large influx of resources to combat a disease considered to have a minor livelihood impact in comparison with diseases such as dengue fever (Ear & Burgos, 2009). Nevertheless, avian influenza projects have generated data on zoonosis transmission at the livestock-human interface and thus national data on zoonotic influenza are readily available, in contrast to data on other zoonoses. In general, only a fraction of zoonoses in livestock and humans are reported to health services, national statistics on zoonosis are incomplete and measures in zoonosis prevention, detection and control are rarely sufficient (Coker *et al.*, 2011).

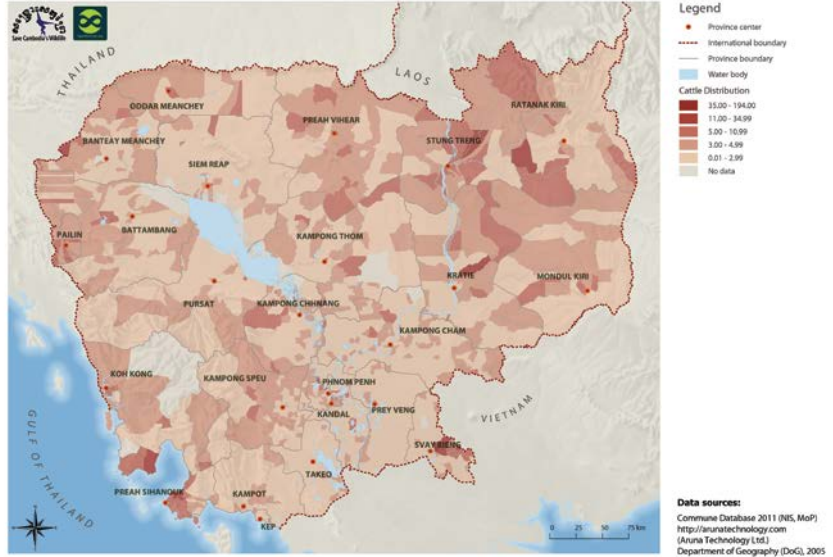
1.4 Household practices and risk factors for zoonoses

In smallholder farming, zoonosis exposure is determined by behavioural factors combined with pathogen characteristics (Randolph *et al.*, 2007). Human social and behavioural factors have a direct effect on R_0 , the basic reproductive rate of a pathogen (defined as the expected number of infections caused by one

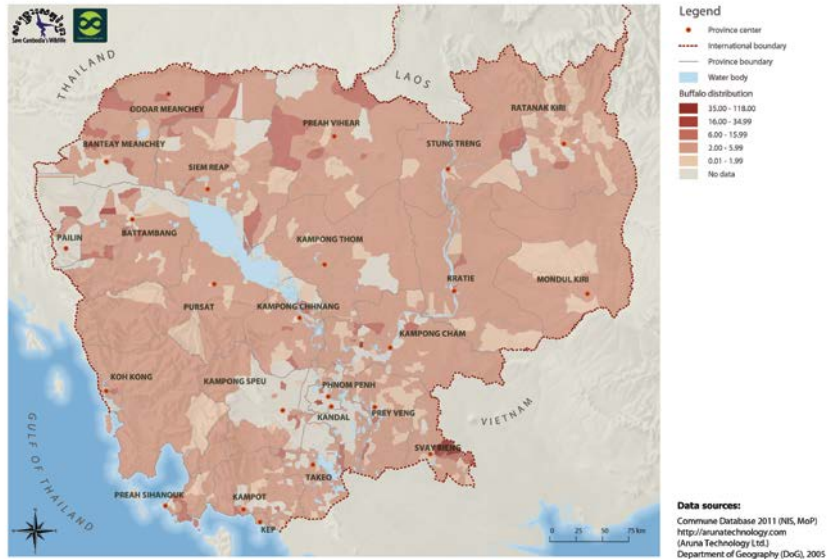
Figure 3. The distribution and density of a) poultry, b) pigs, c) cattle, and d) water buffalo in Cambodia (www.opendevelopmentcambodia.net).



c)



d)



infected individual in a susceptible population) (Janes *et al.*, 2012). R_0 has three components: contact/exposure rate, probability of transmission and duration of infection, which are all partly regulated socially, including factors such as social inequality and poverty (Janes *et al.*, 2012). Social factors linked to zoonosis transmission can be captured by studying household-level behaviour. In the past, various household practices have been identified as risk factors for zoonoses in backyard livestock farming in low-income countries, with the transmission dynamics of the pathogen involved determining the different risks. For the parasite *Toxoplasma gondii*, consumption of undercooked meat has been proposed as a major risk factor for human infection in rural households in Nepal (Petersen *et al.*, 2010). In Egypt, risk factors for bacterial diarrhoea in children from rural households have been identified as presence of poultry manure, uncovered litter in house yards and lack of barriers to keep animals out of houses (Hassan *et al.*, 2014; Rao *et al.*, 2001). Risk factors for human influenza in Cambodia are reported to include inadequate hand washing, slaughtering of poultry and swimming in water frequently used by poultry (Vong *et al.*, 2009).

Numerous development projects have aimed to raise awareness of zoonoses among farmers and to limit zoonosis transmission through improving biosecurity and changing practices. Despite widespread awareness raising, inadequate changes have taken place and follow-up studies have revealed that simply increasing farmers' knowledge may be insufficient to change their behaviour (Alarcon *et al.*, 2014). Additional factors to knowledge and awareness have been proposed to determine and predict human behaviour. Some of these factors may be found in Icek Ajzen's Theory of Planned Behaviour (Ajzen, 1985), which states that human behaviour is guided by three kinds of considerations: beliefs about the likely consequences, which affect the attitude to the behaviour; beliefs about the normative expectations of other people, which affect the social pressure or subjective norm; and beliefs about the presence of factors that may further or hinder performance of the behaviour, which affect the perceived behavioural control. Ajzen suggests that these three pillars (attitudes, subjective norms and perceived behavioural control) are further influenced by various background factors such as affect and emotions (Ajzen, 2011).

The challenge for those wishing to influence farmers' behaviour is to understand the rationality and the social and economic context within which farmers operate. In the past, efforts to control zoonoses among smallholders in low-income countries have devoted less attention to understanding and gaining insights into smallholder drivers and motives for changing their behaviour (Garforth, 2015; Kang'ethe *et al.*, 2012). Moreover, the sources from which

advice and information come may have been underestimated, since recent research has shown that some advice may be rejected simply because a farmer does not consider the person or organisation to be a trustworthy source (Garforth, 2015). A suggested conceptual framework describing farmer behaviour influences is presented in Figure 4.

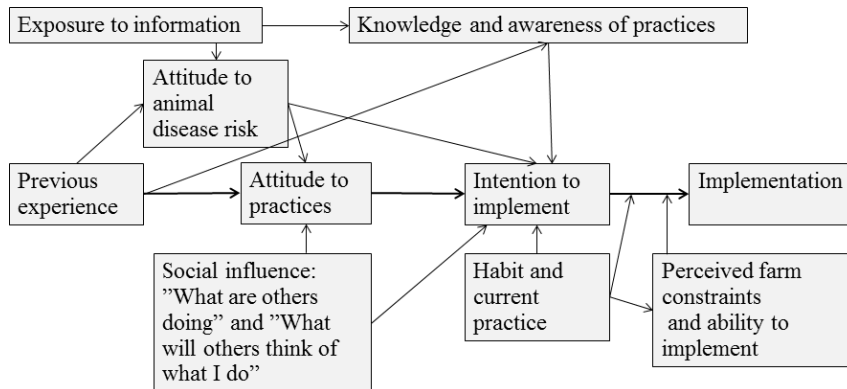


Figure 4. Suggested conceptual framework showing the influences of farmer behaviour, based on the Theory of Planned Behaviour (modified from Garforth, 2015).

1.5 The One Health concept

In the late 1800s, the German physician Rudolf Virchow wrote:

Between animal and human medicine there is no dividing line, nor should there be. The object is different but the experience obtained constitutes the basis of all medicine.

The idea that all animal species, including humans, are related and that knowledge gained in one species benefits all has advanced since it was first introduced by Virchow and has led to the concept of ‘One Health’ (Osburn *et al.*, 2009).

The One Health concept is a worldwide strategy for expanding interdisciplinary collaborations and communications in all aspects of healthcare for humans, animals and the environment. Since the late 1990s, the concept has gained momentum and has become an established term in global health. The term ‘One World-One Health’ was first introduced by the Wildlife Conservation Society in New York in 2004 and, in the wake of the avian influenza pandemic threat, was further developed in joint work by the World

Health Organization (WHO), the Food and Agriculture Organization (FAO) and the World Organization for Animal Health (OIE), leading to publication of the document: ‘*One World-One Health: A strategic framework for reducing risks of infectious diseases in the animal-human-ecosystems interface*’ (Zinsstag *et al.*, 2015; Gibbs, 2014).

Although One Health has been extensively adopted by governments, education institutions and societies, defining the boundaries of the concept remains difficult. At its heart, One Health promotes health through joint interdisciplinary forces taking a societal perspective rather than only a public health perspective (Zinsstag *et al.*, 2015).

1.6 Influenza virus

Outbreaks of influenza have most likely occurred periodically in animals and humans throughout history. ‘Epidemic fever’ was first described by Hippocrates in 412 BC (Monto *et al.*, 2012; Kuszewski & Brydak, 2000). Between 1889 and 1892, during the first confirmed global pandemic, the German scientist Pfeiffer claimed that a germ which he named *Bacillus influenza* was the infectious agent (Pfeiffer, 1931). In the early 1920s, in parallel with the 1918-1920 ‘Spanish flu’ pandemic, influenza was successfully recognised as a viral agent of disease in chickens and pigs (Webster *et al.*, 1992; Shope, 1931). It was not until 1933, however, that the virus was first isolated in humans (Smith *et al.*, 1933). In the 1970s, wild birds were identified as an important reservoir of influenza virus (Hinshaw & Webster, 1982).

1.6.1 Features of influenza virus

Influenza viruses belong to the family Orthomyxoviridae and are classified into four types: A, B, C and D, based on the identity of major internal protein antigens. Influenza A viruses infect humans and multiple mammalian species, influenza B viruses infect humans and occasionally seals and influenza C viruses infect humans, pigs and dogs (Webster *et al.*, 1992). Influenza D virus was identified as a new genus within the Orthomyxoviridae family in 2013 (Hause *et al.*, 2014). The virus has been isolated from swine exhibiting severe influenza-like illness in North America, and from diseased cattle in China, France and North America (Collin *et al.*, 2015; Ducatez *et al.*, 2015; Jiang *et al.*, 2014). Influenza C is generally responsible for sporadic infections in humans, causing mild illness. Influenza B may cause morbidity and mortality in humans but is often associated with less severe epidemics, including seasonal influenza (Pringle, 2016). Influenza A also causes seasonal epidemics, but in addition may cause severe disease and larger outbreaks in humans and

animals (Webster *et al.*, 1992). Unlike influenza B, C and D, influenza A has caused several pandemics in the past.

The influenza A virus genome consists of eight negative-stranded RNA segments that encode different proteins (Table 1). Some of the proteins are always present, some are newly discovered and appear to be present only in certain subtypes. Segment 1 encodes basic polymerase 2 (PB2); segment 2 encodes the polymerase basic (PB) proteins PB1, PB1-F2 and PB1-N40 by using alternative translation initiation sites; segment 3 encodes the polymerase acidic (PA) proteins PA and PA-X by a ribosomal frameshift, as well as two additional N-terminally truncated forms (PA-N155 and PA-N182) by using alternative translation initiation sites; segment 7 encodes the matrix (M) protein M1 and ion channel proteins M2 and M42; and segment 8 encodes the nonstructural (NS) protein NS1 and nuclear export proteins (NEP, sometimes called NS2) NS1-NEG8 and NS3 by alternative mRNA splicing (Selman *et al.*, 2012; Wise *et al.*, 2012; Zhirnov *et al.*, 2007; Dronamraju, 2004). Nuclear export protein (NEP/NS2) is present in virions in low amounts and is thus considered a structural protein.

Influenza A viruses (IAV) are classified into subtypes on the basis of antigenic analysis of the haemagglutinin (HA) and neuraminidase (NA) glycoproteins. So far, 18 subtypes of the HA gene and 11 subtypes of the NA gene have been detected (Tong *et al.*, 2013; OIE, 2012). Wild waterfowl and shorebirds, natural reservoirs of IAV, may harbour combinations of at least 16 different HA subtypes and nine different NA subtypes (Short *et al.*, 2015). The new subtypes H17N10 and H18N11 have recently been found in bats (Tong *et al.*, 2013; Tong *et al.*, 2012).

Influenza A viruses are highly flexible pathogens. They have evolved in association with their various hosts in different continents for long periods of time (Olsen *et al.*, 2006), and the segmented nature of the IAV genome drives the evolution by a process whereby genes from two or more influenza viruses can be mixed. This process is called reassortment and occurs when two distinct influenza strains co-infect a host, resulting in a new strain, as illustrated in Figure 5. In addition, numerous mutations are generated during replication, as IAV polymerase lacks proofreading and post-replication repair mechanisms (Munoz *et al.*, 2015). The evolution is believed to be further promoted by the structural variation between species and the neutralising antibody response of the hosts (Munoz *et al.*, 2015). Thus by scrutinising the influenza genomic structure mechanisms, its spread and disease pathogenesis may be revealed.

Table 1. *Influenza A virus proteins and their functions (S = segment). Modified from (Munoz et al., 2015) with additions from (Selman et al., 2012; Wise et al., 2012; Zhirmov et al., 2007)*

S	Coded protein	Abbreviation	Stage of cycle	Function
1	Basic polymerase 2	PB2	Replication	Viral RNA synthesis
2	Basic polymerase 1	PB1	Replication	Viral RNA synthesis
	PB1-F2		Host immune response	Pro-apoptotic activity and inclusion of a pro-inflammatory response
	PB1-N40		Unclear	Unclear
3	Acid polymerase	PA	Replication	Viral RNA synthesis
	PA-X		Host immune response	Probable host gene expression modulation
	PA-N182		Unclear	Unclear
	PA-N155		Replication	Unclear
4	Haemagglutinin	HA	Attachment, cell entry	Virus binding and fusion of the viral endosomal membranes
5	Nucleocapsid protein	NP	Release	Viral RNA synthesis
6	Neuraminidase	NA	Replication	Cleaves residues, promotes release and prevents viral particle aggregation
7	Matrix protein	M1	Release	Involved in export from host cell nucleus, viral packaging and budding
	Ion channel protein	M2	Cell entry	Transmembrane ion channel
	M42		Unclear	Unclear
8	Non-structural protein	NS1	Host immune response	Antagonises the innate immune response
	NS3		Unclear	Unclear
	NS1-NEG8		Unclear	Unclear
	Nuclear export protein	NEP/NS2	Release	Involved in export from host cell nucleus

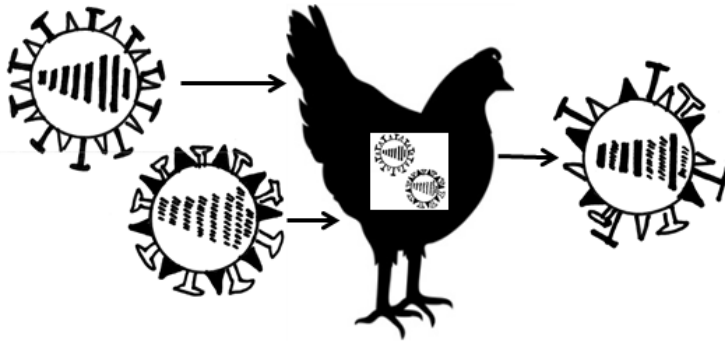


Figure 5. Reassortment of two influenza A viruses in a chicken.

1.6.2 Influenza A virus in livestock

Domestic animal populations, especially birds and pigs, represent an important source of multiple diverse genotypes of IAVs. The primordial reservoirs of influenza viruses, however, are considered to be wild aquatic birds (Olsen *et al.*, 2006). Quails and turkeys have been proposed as disease amplifiers and bridging species for transmission of IAV from wild birds to domestic poultry (Munoz *et al.*, 2015). In birds, influenza disease signs vary considerably with the infected avian species and the IAV subtype and strain (Capua & Alexander, 2007). Strains of avian influenza virus (AIV) are classified into low pathogenic (LPAI), causing asymptomatic to mild respiratory disease and depression, and the very virulent highly pathogenic (HPAI) strains which, when introduced into poultry, often cause systemic infection and death (Olsen *et al.*, 2006). The latter has been restricted to members of the H5 and H7 subtypes (Webster *et al.*, 1992).

Although long known as fowl plague, HPAI was first described in 1959. During the past 20 years, there has been an alarming increase in the number of outbreaks and birds involved (Capua & Alexander, 2007). Highly pathogenic (HP) H5N1 arose among domestic geese in China in 1996 and several lineages of this virus have established endemic and epidemic infections throughout Asia and Africa following multiple reassortment events (Shortridge *et al.*, 1998). In parallel, other subtypes of AIV with zoonotic potential have emerged in Asia. Most notable is H9N2, which has caused extensive outbreaks in poultry and is considered an important gene donor to other influenza viruses (Sun & Liu, 2015), and H7N9, which circulates asymptotically in poultry in China but causes severe influenza in humans (Peiris *et al.*, 2015).

In pigs, influenza is mainly a respiratory disease producing signs of fever, cough and pneumonia, often as a contributing pathogen to the porcine respiratory disease complex (Vincent *et al.*, 2014). Three subtypes: H1N1,

H1N2 and H3N2, are circulating globally in pigs and most of the currently circulating strains are human/avian/swine reassortants carrying human-derived HA (Munoz *et al.*, 2015). In the late 1990s, the epidemiology of swine influenza virus (SIV) changed dramatically as new reassortants of SIV with internal genes from avian, human and swine IAV, named triple reassortant (TR) SIV, were detected in North America (Vincent *et al.*, 2014). The TR SIV has PA and PB2 segments from AIV, PB1 from human influenza virus and NP, M and NS segments from the classical H1N1 SIV (Munoz *et al.*, 2015). Since its discovery it has spread to Asia, where it has been detected in South Korea, China and Vietnam (Baudon *et al.*, 2015; Lyoo *et al.*, 2014; Ngo *et al.*, 2012; Fan *et al.*, 2011). During their long-term evolution, both AIV and SIV have divided into Eurasian and American lineages (Garten *et al.*, 2009; Olsen *et al.*, 2006).

1.6.3 Influenza A virus in humans

In humans, influenza A may cause respiratory disease characterised by sudden onset of high fever, cough, headache, malaise and inflammation of the upper respiratory tract. People of all ages are afflicted, but the prevalence is highest in school-age children and disease severity is greatest in infants, the elderly and those with an underlying illness (Taubenberger & Morens, 2008). Measures to control human influenza include vaccination or antiviral drugs administered prophylactically or therapeutically (Couch, 2000). The most commonly used antivirals for influenza treatment are the NA inhibitors zanamivir (RelenzaTM) and oseltamivir (TamifluTM), and the M2 inhibitors amantadine (SymmetrelTM) and rimantadine (FlumadineTM), but newer antivirals are being introduced to override the emergence of drug-resistant viruses (Gubareva, 2004).

The WHO differentiates between seasonal, pandemic and zoonotic influenza in humans (WHO, 2014). Seasonal influenza circulates and causes disease in humans annually and may be isolated year-round in tropical and subtropical regions of the world, whereas in temperate climates influenza is mainly a winter disease (Webster *et al.*, 1992). Pandemic influenza occurs when an influenza virus which was not previously circulating among humans, and to which most people do not have immunity, emerges and transmits among humans (WHO, 2014). Zoonotic influenza occurs when humans are infected with influenza viruses that are routinely circulating in animals, such as avian and swine influenza subtypes. Such human infections are usually acquired through contact with infected animals or contaminated environments and seldom spread far among humans. However, zoonotic influenza may cause an

epidemic or a pandemic if the virus acquires the capacity for human to human transmission (WHO, 2014).

In the past century there have been four major pandemics of varying severity and origin (Table 2): the 1918 H1N1 ‘Spanish flu’, the 1957 H2N2 ‘Asian flu’, the 1968 H3N2 ‘Hong Kong flu’ and the 2009 H1N1 ‘pdm09’ (Short *et al.*, 2015; Kuszewski & Brydak, 2000). Some of the pandemic viruses have since become extinct, while others have resurged after a few years or have become established in the human population and are responsible for seasonal influenza (Short *et al.*, 2015). All four pandemics are suggested to have been generated through a series of reassortment events in mammals over a period of years before successful adaptation to humans (Smith *et al.*, 2009a; Smith *et al.*, 2009b).

Table 2. Influenza pandemics during the past century (WHO, 2011)

Years	Name	Subtype	Extent of outbreak
1918-1919	Spanish flu	H1N1	20-50 million deaths
1957-1958	Asian flu	H2N2	1-4 million deaths
1968-1969	Hong Kong flu	H3N2	1-4 million deaths
2009-2010	pdm09	H1N1	18 500 deaths laboratory confirmed

1.6.4 Zoonotic potential of influenza A virus

Each year, IAV infects 5-10% of the human population and millions of poultry and pigs (WHO, 2016b). Several IAVs have potential for bi-directional transmission between humans and animals, a route most commonly detected to date between humans and pigs (Munoz *et al.*, 2015). The risk of human infection by a zoonotic IAV depends on the dynamic interplay between the virus, environmental factors and the immune system of the human host. Infections with avian or swine influenza viruses in humans occur sporadically (Schrauwen & Fouchier, 2014) and identified risk factors for human AIV infection (mainly HP H5N1) in rural households include direct/indirect contact with sick/dead poultry, visits to wet markets, cleaning of faeces and proximity to/swimming in ponds (Vong *et al.*, 2009; Zhou *et al.*, 2009b). The first documented case of direct AIV transmission from poultry to humans occurred in Hong Kong in 1997 and more have followed since. To date, three HA subtypes (H1, H2 and H3) have been shown to have the ability to transmit efficiently among humans and five HA subtypes (H5, H6, H7, H9 and H10) have infected humans after crossing the inter-species barrier (Figure 6) (Schrauwen & Fouchier, 2014). Fortunately, these zoonotic influenza viruses so far lack the ability to spread efficiently between humans.

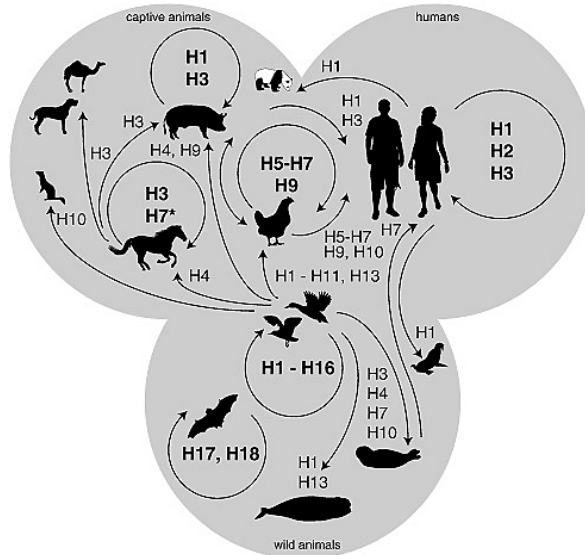


Figure 6. Reservoirs and inter-species transmission events of avian influenza viruses. Wild birds, domestic birds, pigs, horses, humans and bats maintain their own influenza A viruses (arrow in circle, subtype in bold). Spill-over events occur occasionally, most frequently from wild birds (arrow straight, subtype normal font). Original picture used as available under the terms of the Creative Commons Attribution License (CC BY) (Short *et al.*, 2015).

As regards SIV, in recent years both classical SIVs and TR SIVs have been isolated in humans (Garten *et al.*, 2009). Pigs are considered a ‘mixing vessel’ for avian and mammalian IAVs, as they possess receptors for both virus types. The scientific basis and the extent to which pigs contribute to the emergence of novel and pandemic strains are not clear, however (Munoz *et al.*, 2015). Nevertheless, it is feared that SIV or AIV may mutate or reassort with circulating human influenza viruses, possibly resulting in better adaptation to humans and subsequent human-to-human transmission (Neumann & Kawaoka, 2015).

1.6.5 Influenza A virus in Cambodia and Southeast Asia

Southeast Asia has been severely affected by IAV, in particular HP H5N1, resulting in the culling of more than 175 million birds and 463 human cases with 315 deaths (Pfeiffer *et al.*, 2013, WHO, 2016a). H5 subtype viruses, in particular clade 2.3.4.4, have also shown a predilection for genetic reassortment, giving rise to the H5N2, H5N5, H5N6 and H5N8 virus subtypes. These emerging subtypes have caused infections in wild birds and poultry globally and throughout Southeast Asia, and H5N6 has been confirmed in three human cases in China (Mok *et al.*, 2015). Increasing numbers of subtypes other

than H5 have also emerged in Southeast Asia, such as the previously mentioned H9N2 and H7N9 (Peiris *et al.*, 2015; Sun & Liu, 2015).

In contrast to surrounding countries and for unknown reasons, Cambodia has a southern hemisphere transmission pattern of seasonal human influenza that occurs during June-December each year (Mardy *et al.*, 2009). However, human cases of HPAI and other IAVs viruses occur during the dry season (November-April) and coincide with HP H5N1 outbreaks in poultry (Horm *et al.*, 2014). The temporal peaks in HP H5N1 correspond with national festivals at the beginning of the year (Chinese/Vietnamese New Year in February and Khmer New Year in April) and the release of ducklings into rice fields after harvest in the south (Buchy, 2014).

Highly Pathogenic Avian Influenza is considered epizootic in Cambodia. Since the first detection in 2004, the government of Cambodia, with support from international organisations and NGOs, has run massive public awareness campaigns about HPAI and have imposed control measures for poultry, including poultry movement restrictions, culling of infected flocks (without economic compensation), surveillance zones around outbreaks and temporary suspension of sales and purchases of birds (Burgos *et al.*, 2008). Nevertheless, despite the efforts to contain H5N1, it remains epizootic and since 2004 Cambodia has reported 42 poultry outbreaks and 56 human cases (OIE, 2016; WHO, 2016a). Influenza vaccination is prohibited in poultry and negligible in pigs in Cambodia and in comparison with neighbouring Vietnam and Thailand, Cambodia is considered less severely affected by H5N1, but it has been suggested that minor poultry outbreaks and less severe human cases are underreported (Wang *et al.*, 2012).

Several emergences of SIV have been distinguished in Southeast Asia, with evidence of multiple introductions of H1 North American and avian-like H1 European strains. The subtypes reported have mainly been H3N2 and H1N1, but Southeast Asia is the only region in the world where pig infections with avian-origin H5N1 and H9N2 have been reported (Trevennec *et al.*, 2011). Several studies have isolated H3N2 in pigs in Vietnam (Baudon *et al.*, 2015; Ngo *et al.*, 2012), but to date no influenza virus has been isolated from Cambodian pigs, although antibodies to human H1N1 and H3N2 have been detected (Netrabukkana *et al.*, 2014; Rith *et al.*, 2013).

1.7 *Campylobacter*

In 1886, the German paediatrician Theodor Escherich published a series of articles in which he described spiral bacteria in the colon of children who had died of what he called '*cholera infantum*' (Escherich, 1886). Twenty-three

years later, British veterinarians reported an unknown bacterium frequently isolated from aborted lambs (McFadyean, 1909). It was not until the 1970s, however, that *Campylobacter* was successfully isolated from human faeces and recognised as an important human pathogen (Butzler, 2004).

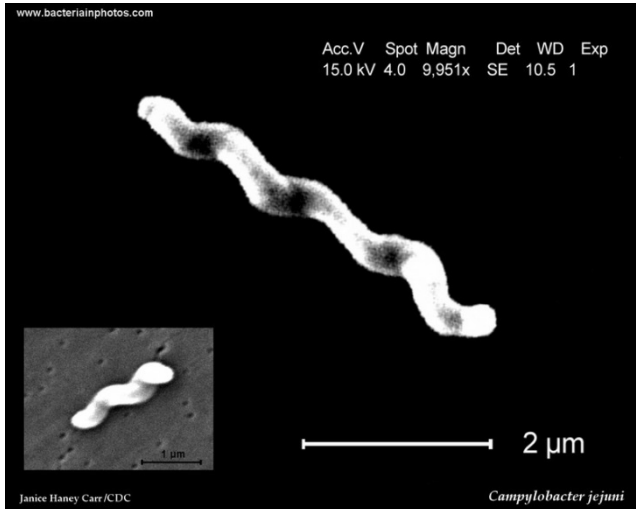


Figure 7. Electron microscope image of *Campylobacter jejuni* (photo: Janice Haney Carr/CDC).

1.7.1 Features of *Campylobacter* bacterium

The taxonomy of the family Campylobacteraceae has evolved extensively during the past 50 years, but currently comprises the genera *Campylobacter* (30 taxa), *Arcobacter* (17 taxa) and *Sulfurospirillum* (seven taxa). All *Campylobacter* spp. are non-spore forming, Gram-negative microaerophiles (Lastovica *et al.*, 2014). The bacteria (0.2-0.8 x 0.2-5.0 μ m) of most species are curved and move with a characteristic screw-like movement by means of a flagellum (Nachamkin *et al.*, 2008; Humphrey *et al.*, 2007) (Figure 7).

Some *Campylobacter* spp. are ubiquitous in the environment and have been isolated from soil and mud (Lastovica *et al.*, 2014). They have also been commonly detected in untreated drinking water (Domingues *et al.*, 2012). *Campylobacter* spp. can colonise the mucosal surfaces in humans and a wide range of wild and domesticated birds and mammals. Some species cause infections of the reproductive tract, while others are involved in gastrointestinal and periodontal diseases (Humphrey *et al.*, 2007). A few species are host associated, but most can colonise various hosts (Lastovica *et al.*, 2014) (see Table 3).

1.7.2 *Campylobacter* in livestock

In animals, the clinical importance of *Campylobacter* infection is mainly attributed to the species *Campylobacter fetus*, which was most likely the first *Campylobacter* isolated (McFadyean, 1909). The species comprises two subspecies, *C. fetus* subsp. *fetus* and *C. fetus* subsp. *veneralis*. *Campylobacter fetus* subsp. *fetus* have been isolated from a range of species, including fowl, reptiles and humans, but is mainly associated with abortion in sheep and cattle. The pathogen has also been detected in immunodeficient human patients and in neonatal sepsis and septic abortions. *Campylobacter fetus* subsp. *veneralis* causes bovine genital campylobacteriosis and infectious venereal disease that may lead to infertility, abortion and embryo death (Nachamkin *et al.*, 2008).

A high carriage rate of *Campylobacter* as an asymptomatic coloniser is found in a wide range of birds and mammals and is considered a public health threat when occurring in livestock and pets (Kaakoush *et al.*, 2015). *Campylobacter* spp. are commonly found as colonisers in the intestinal tract in poultry, pigs and cattle, mainly the species *C. jejuni* and *C. coli*, but also *C. upsaliensis*, *C. concisus*, *C. lari* and *C. lanienae*. Once *Campylobacter* is introduced into a flock, it can spread rapidly. In poultry it generally results in life-long colonisation (Kaakoush *et al.*, 2015).

1.7.3 *Campylobacter* in humans

In humans, *Campylobacter* are known mainly for causing enteritis and are considered the most common pathogen causing bacterial gastroenteritis worldwide (WHO, 2015; WHO, 2013). Most infections are caused by the thermophilic zoonotic species *C. jejuni* and *C. coli* (Lastovica *et al.*, 2014) and the infectious dose has been reported to be as low as 500 bacteria (Black *et al.*, 1988).

The onset of clinical symptoms following intake of *Campylobacter* usually occurs 24 to 72 h post-ingestion and the symptoms range from acute abdominal pain, diarrhoea and fever to late sequelae such as reactive arthritis and the rarely occurring neurological Guillain-Barré syndrome (Kaakoush *et al.*, 2015; Janssen *et al.*, 2008). Acute *Campylobacter* enteritis has also been linked to irritable bowel syndrome and inflammatory bowel disease (Kaakoush *et al.*, 2015; Havelaar *et al.*, 2009). The signs of *Campylobacter* infection are not characteristic and it is impossible to clinically differentiate infection by this pathogen from illnesses caused by other gastrointestinal pathogens (Butzler & Oosterom, 1991).

Table 3. Sources and disease associations of *Campylobacter* species in livestock and humans (modified from (Lastovica et al., 2014; Humphrey et al., 2007))

Species or subspecies	Known host(s)	Disease associations	
		Human	Animal
<i>C. avium</i>	Chickens, turkeys	Unknown	Unknown
<i>C. coli</i>	Pigs, poultry, cattle, sheep, birds, humans	Gastroenteritis, septicaemia	Gastroenteritis
<i>C. concisus</i>	Dogs, cats and humans	Periodontal disease, gastroenteritis, septicaemia, infectious bowel disease	Gastroenteritis
<i>C. cuniculorm</i>	Rabbits	Unknown	Unknown
<i>C. curvus</i>	Humans	Gastroenteritis, abscesses, periodontal disease	Unknown
<i>C. fetus</i> subsp. <i>fetus</i>	Cattle, sheep, dogs, turtles	Septicaemia, meningitis, vascular infection, abortion	Spontaneous abortion (cattle, sheep)
<i>C. fetus</i> subsp. <i>veneralis</i>	Cattle	Septicaemia	Bovine infectious infertility
<i>C. gracilis</i>	Dogs, humans	Abscesses, periodontal disease, emphysema	Unknown
<i>C. helveticus</i>	Dogs, cats, humans	Unknown	Gastroenteritis
<i>C. hominis</i>	Humans	Gastroenteritis in immunocompromised individuals	Unknown
<i>C. hyointestinalis</i> subsp. <i>hyointestinalis</i>	Pigs, cattle, hamsters, deer	Gastroenteritis, septicaemia	Gastroenteritis (pigs, cattle)
<i>C. hyointestinalis</i> subsp. <i>lawsonii</i>	Pigs, poultry, birds	Unknown	Unknown
<i>C. hyoilei</i>	Pigs	Unknown	Porcine proliferative enteritis

Species or subspecies	Known host(s)	Disease associations	
		Human	Animal
<i>C. jejuni</i> subsp. <i>jejuni</i>	Dogs, cattle, birds, poultry, sheep, shellfish, humans <i>etc.</i>	Gastroenteritis, septicaemia, myocarditis, arthritis <i>etc.</i>	Spontaneous abortion (cattle, sheep), gastroenteritis (dogs, cats)
<i>C. jejuni</i> subsp. <i>doylei</i>	Dogs, humans	Enteritis, septicaemia	Unknown
<i>C. lanienae</i>	Cattle, pigs, humans	Unknown	Unknown
<i>C. lari</i> subsp. <i>lari</i>	Cats, dogs, chickens, seals, mussels, oysters	Gastroenteritis, septicaemia	Avian gastroenteritis
<i>C. mucosalis</i>	Pigs, dogs	Unknown	Porcine necrotic enteritis and ileitis
<i>C. peloridis</i>	Molluscs, humans	Unknown	Unknown
<i>C. rectus</i>	Humans	Abscesses, periodontal disease	Unknown
<i>C. showae</i>	Dogs, humans	Septicaemia, cholangitis, periodontal disease	Unknown
<i>C. sputorum</i> bv. <i>paraureolyticus</i>	Cattle, humans	Unknown	Unknown
<i>S. sputorum</i> bv. <i>faecalis</i>	Cattle, sheep	Unknown	Unknown
<i>S. sputorum</i> bv. <i>sputorum</i>	Cattle, pigs, sheep, humans	Abscesses, gastroenteritis	Unknown
<i>C. upsaliensis</i>	Cats, dogs, ducks, monkeys	Gastroenteritis, septicaemia, abscesses	Gastroenteritis (dogs, cats)
<i>C. ureolyticus</i>	Cattle, humans	Ulcerative colitis	Unknown

The epidemiology of human *Campylobacter* infection is markedly different in tropical and temperate countries. Infection occurs year-round in tropical countries, whereas in temperate countries infection has summer and autumn peaks (Nielsen *et al.*, 2013). More important, however, is the distribution of infection in different age groups. In high-income countries, infection is often symptomatic and all age groups are at risk, although infection is suggested to be more prevalent in toddlers (1-4 years) and young adults (Nielsen *et al.*, 2013). In low-income countries, symptomatic infection is usually limited to children below 5 years, with illness/infection ratio decreasing with age (Kaakoush *et al.*, 2015). Adults and older children rarely fall ill, as repeated exposure to a wide range of *Campylobacter* strains at a young age leads to protective immunity to clinical disease, but not necessarily to asymptomatic re-infection (Havelaar *et al.*, 2009). The clinical picture also differs from that seen in high-income countries, with watery diarrhoea being the most common presentation in low-income countries (Havelaar *et al.*, 2009). In resource-poor settings, children are also considered at higher risk of developing serious forms of bacterial diarrhoeal diseases due to malnutrition. Gastrointestinal infections in turn exacerbate malnutrition, thus leading to a vicious circle of debilitation and mortality (WHO, 2015).

1.7.4 Zoonotic potential of *Campylobacter*

In the majority of *Campylobacter* infections reported to date, transmission to humans occurred through direct contact with livestock or through consumption of contaminated meat, milk or water (EFSA, 2015; Domingues *et al.*, 2012). Poultry is recognised as the primary source and 50-80% of all human *Campylobacter* infections are suggested to be related to chicken (EFSA, 2015). However, the number of human *Campylobacter* infection cases attributed to chicken is higher than the number estimated to be acquired through consumption of chicken meat. Thus infection may result from indirect transmission from chickens, and not only through the usual eating or handling routes (Nichols *et al.*, 2012). Interestingly, in high-income countries international travel, in particular to Southeast Asia, is suggested to be the most important risk factor for campylobacteriosis (Domingues *et al.*, 2012).

1.7.5 *Campylobacter* in Southeast Asia

An unusually high incidence of campylobacteriosis has been reported in certain regions of Asia and campylobacteriosis is considered endemic throughout Southeast Asia, especially in children (Kaakoush *et al.*, 2015). Studies in rural areas in Thailand and Vietnam have reported *Campylobacter* as the most

frequently occurring bacterial cause of diarrhoea in children (Bodhidatta *et al.*, 2010; Isenbarger *et al.*, 2001). However, the only known publication on campylobacteriosis in Cambodia identified rotavirus and enteroaggregative *Escherichia coli* as the major causes of acute diarrhoea in children enrolled at the National Pediatric Hospital in Phnom Penh (2004-2006). *Campylobacter jejuni* was detected in 4.7% of children with diarrhoea and *C. coli* in 1.5%, and *Campylobacter* were equally or more common in the healthy controls than in cases (Meng *et al.*, 2011).

Studies on *Campylobacter* positivity in livestock in Southeast Asia are equally scarce. A recent study on livestock in Vietnam detected *Campylobacter* in 32% of poultry and 54% of pigs sampled on low-biosecurity farms (Carrique-Mas *et al.*, 2014). In a rural market in Thailand, *Campylobacter* were detected in 93% of raw chicken samples and in 17% of raw pork samples (Bodhidatta *et al.*, 2013). In Cambodia, 81% of the poultry carcasses available for sale in markets tested positive for *Campylobacter* by culture (Lay *et al.*, 2011). To the best of my knowledge, there are no previous publications on detection of *Campylobacter* in humans and livestock on Cambodian smallholdings.

2 Aims of the thesis

The overall objective of this thesis was to provide science-based information on the interplay between humans and livestock in rural tropical areas in general and in Cambodia in particular, using epizootic influenza A virus and endemic *Campylobacter* bacteria in rural livestock-keeping households as examples of zoonotic pathogens.

Specific aims were to:

- Describe livestock management practices on rural smallholder farms in three different agro-ecological regions of Cambodia.
- Relate practices known to be associated with zoonotic disease transmission to the household's agro-ecological region, socio-economic position, number of livestock and people, livestock management and zoonosis awareness.
- Determine the prevalence and genetic characteristics of influenza A virus in backyard-farmed pigs and poultry.
- Assess the performance of routine culturing and multiplex PCR to detect *Campylobacter* in livestock and human faecal samples collected in the field.
- Estimate the prevalence of *Campylobacter jejuni* and *Campylobacter coli* in livestock and humans.
- Identify zoonotic risk factors associated with *Campylobacter jejuni* and *Campylobacter coli* positivity in humans.

3 Considerations on materials and methods

This section provides a summary and comments on the material and methods used in Papers I-IV. Detailed descriptions of the procedures performed are presented in the individual papers.

3.1 Study sites and study design

The studies described in Papers I-IV included households in the same villages and provinces in Cambodia. To cover possible differences in climate and farming traditions, three of Cambodia's four agro-ecological regions were involved, with exclusion of the mountainous region (NIS, 2013). For increased possibility of detecting influenza virus [Paper II], samples were collected at the end of the dry season or beginning of the rainy season, in provinces exposed to repeated outbreaks of H5N1 in poultry and humans (OIE, 2016; WHO, 2016a). Kampong Cham province in the plains region was visited in May 2011, Battambang province in the wetlands region in July 2012 and Kampot province in the coastal region in March 2013 (Figure 8).



Figure 8. Map of Cambodia and picture of field team members wearing project T-shirts.

Ten villages were selected for inclusion in each region. The number of villages was based on practical and economic considerations for sample collection, with the specific selection criteria: the village had to be situated within 5 km from a main road; it had to have various species of livestock; and there had to be interactions between humans, domestic animals and wildlife. Within each village, the 10 households keeping as many different livestock species as possible, according to the village animal health worker and village head, were selected as a purposive sample. The target number of households and samples were based on sample size for expected influenza and *Campylobacter* prevalence in humans and livestock [specified in Papers II and IV], at 95% confidence interval (CI) and 5% precision, using the formula presented by Thrusfield (Thrusfield, 2007). Due to the estimated average of three livestock and human samples per household, target sample size was further adjusted for intra-cluster correlations, with a coefficient of 0.2 (Dohoo *et al.*, 2003; Otte & Gumm, 1997).

Simple random sampling of villages and households would have been optimal from a statistical perspective, but was impractical given the logistical arrangements for sample collection and transport. However, there is no reason to suspect selection bias of villages and households and therefore the results can be assumed to serve as an approximation of a population-based design for households rearing multiple species of livestock.

A cross-sectional study design was used in all four studies and each village was visited twice, on day one to carry out interviews and distribute materials for human self-sampling [Papers I-IV] and on the following day to collect human and livestock samples and fill in sampling forms [Papers II-IV]. Data were collected by a survey team of 10-12 members including national and local livestock officers and university students. Some members of the team were the same for the three provinces and each team member was trained for one full day prior to the fieldwork, to enhance consistency between collection rounds. Before the interviews, participating households were informed about the study objectives and that participation was voluntary. After verbal consent had been received, the household was allocated a code to conceal the identity of participating household members. Towards the end of the fieldwork, all households joining were given a project T-shirt and a bar of soap as a gift for their involvement. A picture of the project T-shirts can be found in Figure 8.

Ethical approval (43 NECHR, 8 April 2011) was obtained prior to the fieldwork from the National Ethics Committee for Health Research, Ministry of Health, Cambodia, and an advisory ethical statement (Dnr 2011/63) was obtained from the Regional Board for Research Ethics in Uppsala, Sweden.

3.2 Questionnaires

In Papers I, II and IV, information from questionnaires was used. Two separate questionnaires were developed: i) a village questionnaire targeted at the village head and animal health worker, with questions on village development support and livestock management [Paper I]; and ii) a household questionnaire, targeted at the female head of the household, with questions on household practices related to zoonosis transmission (Table 4), socio-economic position, number of livestock and humans, livestock management, meat consumption and zoonosis awareness [Papers I, II and IV]. Household interviews targeted the female head as Cambodian women, to a larger extent than men, are involved in day-to-day household duties and subsistence farming (Resurreccion, 2006). In practice, however, it proved difficult to exclude men during the interviews and in about one-third of the households the male and female head both participated as illustrated in Figure 9. The interviews were carried out in the Khmer language, using Khmer versions of the questionnaires that were pre-tested and adjusted according to input before the study begun. Questions were open, closed and semi-closed, with some probing and validation questions to clarify answers and allow the questionnaires to be checked for internal consistency. After completion of the fieldwork, data collected were translated from Khmer into English by two independent translators and compared for consistency. Measures were taken to reduce bias in the interviews (such as the questionnaire pre-testing, validation questions and double translations) and the

Table 4. *Self-reported household practices in the 300 Cambodian households interviewed*

Which of these practices do you employ in this household? (n=300)	Number (%)
Eat undercooked meat	23 (8)
Feed livestock uncooked meat waste	55 (18)
Cull sick animals for consumption	83 (28)
Eat animals found dead	85 (28)
Allow animals in sleeping and food preparation areas	85 (28)
Slaughter domestic animals	191 (64)
Capture and slaughter wild animals for consumption	24 (8)
Wash hands with soap before and after cooking	268 (89)
Wash hands with soap after handling live animals	251 (84)
Bury or burn meat waste products	242 (81)
Collect manure indoors and outdoors daily	260 (87)



Figure 9. Interviewing one of the households in Kampong Cham province.



Figure 10. Demonstration of tracheal influenza sampling in chickens.



Figure 11. The field team in Battambang province.

information obtained can be regarded as true and valid, although it is likely that some respondents gave what they perceived as desirable answers, which may have influenced the responses.

3.3 Sample collection and transport

In Papers III and IV, faeces samples from humans and livestock (chickens, ducks, pigs, cattle, water buffalo, quail, pigeons and geese) were collected and placed in vials with bacterial freeze medium. Fresh faecal material was collected on swabs from livestock, whereas human samples were provided in plastic containers and transferred with swabs into duplicate vials by the survey team. To optimise survival of *Campylobacter* bacteria, immediate swabbing of humans would have been preferred, but was discouraged by our Cambodian project partners owing to cultural inappropriateness.

In Paper II, cloacal and tracheal swabs were collected from chickens (Figure 10) and ducks, swabs with fresh faecal material from pigeons and nasal swabs from pigs by the field team (Figure 11). Each swab was dipped into separate vials of virus transport medium, spotted onto Flinders Technology Associates (FTA) Indicator Four Spots cards (Qiagen, Hilden, Germany) and then placed back into the vial. FTA[®] cards were stored and shipped in room temperature. A picture of the FTA[®] card is found in Figure 12.

For each person and livestock sampled in Papers II-IV, information on age and disease symptoms within a two-week period prior to sampling was recorded in a sampling form (Figure 13). All bacterial and viral swab samples collected were stored and transported on ice in cooler boxes to Phnom Penh on the day of collection (for samples collected in Kampong Cham province) or by public transport the following morning (for samples collected in Battambang and Kampot province). Once in Phnom Penh, samples were placed in -70 °C freezers. The number of samples collected was considered too high to allow immediate analysis at the Cambodian partner laboratories. All samples were therefore frozen.

Vials with livestock samples were stored at the National Veterinary Research Institute (NaVRI) and vials with human samples at the National Institute of Public Health (NIPH) awaiting analysis in Cambodia or shipment to Sweden. Shipment was facilitated on dry ice by a courier company and required an export permit for each of the four shipments out of Cambodia and an import permit for livestock samples arriving Sweden. The approval time of export permits (4-6 months) and the half-a-year general export stop from Cambodia in 2013 prolonged the storage of samples and may have influenced the recovery and detection of *Campylobacter*.

3.4 Detection of influenza A virus [Paper II]

Influenza virus is commonly identified by direct antigen detection, virus isolation in embryonated eggs or cell culture, or detection of influenza-specific RNA using reverse transcriptase-polymerase chain reaction (RT-PCR). Virus isolation is the gold standard and widely adopted, but PCR-based tests are gaining popularity for their fast performance with comparatively higher sensitivity and specificity (Kim & Poudel, 2013).

All influenza samples in Paper II were analysed at the National Veterinary Institute (SVA) in Sweden. The OIE-recommended method of real-time reverse transcription polymerase chain reaction (rRT-PCR) (OIE, 2012) has been validated for influenza detection at SVA, and was therefore selected. Furthermore, influenza A virus was detected and sequenced from the FTA[®] cards (commonly used and assessed at SVA). Swab samples were saved as back-up, but the quality of the FTA[®] card samples proved adequate and thus no swab samples were analysed. All influenza analyses were initially planned to be performed at NaVRI in Cambodia, but the plans were changed due to difficulties handling the samples that arose following a sudden increase in national influenza outbreaks in 2013.

3.4.1 Real-time reverse transcription PCR

Punches of sample material were removed from each FTA[®] card and detection of nucleic acid was performed by rRT-PCR selective for the matrix gene of influenza type A viruses (Spackman *et al.*, 2003; Spackman *et al.*, 2002). All PCR reactions were performed in duplicate with the reported dye (FAM) measured against the internal reference dye (ROX) to account for non-PCR related fluorescence. The threshold-crossing values (Ct) assigned to each sample in the exponential phase of the amplification plot of each cycle were categorised as positive \leq Ct35, suspected 35-40 and negative $>$ 40.

3.4.2 Sequencing and phylogenetic analyses

Samples identified as IAV-positive by rRT-PCR were further analysed by rRT-PCR specific for haemagglutinin gene of H5, H7 and H9 and neuraminidase gene of N1 subtype (Spackman *et al.*, 2002, Monne *et al.*, 2008). Furthermore were the rRT-PCR IAV positive samples selected for complete genome sequencing. The RNA was converted to full-length cDNA using the universal primer corresponding to the 5' and 3' conserved sequences of all eight influenza type A segments (Zhou *et al.*, 2009a; Hoffmann *et al.*, 2001). After sequencing, assembly of sequences and removal of low-quality sequence data, additional multiple sequence alignments and processing were performed. Phylogenetic analysis was carried out for the complete open reading frame of

each segment, using the maximum-likelihood method implemented in the MEGA6 software (Tamura *et al.*, 2013). For the dataset, 2000 bootstrap re-samplings were performed to assess the robustness of each node. The BLAST algorithm (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was applied to compare sequences with those available at the National Center for Biotechnology Information (NCBI). For the nomenclature of detected influenza viruses, the WHO guidelines were followed. First, the type of virus was designated (A, B, C or D), then the host (as non-human), place of isolation, isolation number and year of isolation, all separated by slashes (Taubenberger & Morens, 2008). For example: A/Swine/Cambodia/TangKrang/060301/2011.

3.5 Detection of *Campylobacter* [Papers III and IV]

In the past, the majority of infection studies have used selective culture techniques for *Campylobacter* detection in faecal samples, but several other techniques are gaining ground (Platts-Mills *et al.*, 2014). *Campylobacter* diagnosis using molecular detection with PCR-based methods and antigen-capture-based tests with enzyme immunoassays are faster than culture, less laborious and generally allow detection with higher sensitivity (Humphrey *et al.*, 2007).

Identification of *Campylobacter* to species level can be based on classical phenotypic characteristics, including biochemical tests such as microscopy and hippurate hydrolysis test or by metabolic markers; matrix assisted laser desorption ionization-time of flight (MALDI-TOF); genotyping such as amplified fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE) or nucleotide sequence-based typing (including sequencing of flagellin genes, multi-locus sequence typing (MLST) and next-generation sequencing (NGS)) (Kaakoush *et al.*, 2015, Humphrey *et al.*, 2007).

3.5.1 Culture

When applying selective culture, there is no simple gold standard or common method for the isolation of all *Campylobacter* spp. and several agar media may be used. For enumeration, direct spread plating on selective media (blood based or blood-free charcoal-based) is frequently used (Habib *et al.*, 2011) (Figure 14). Alternatively, plating is preceded by enrichment for recovery of damaged cells or for samples with a small starting number (Habib *et al.*, 2011). In screening of livestock samples, enrichment is routinely applied prior to plating to allow detection of low numbers of *Campylobacter*. In human samples, however, culturing without enrichment is routinely used and is the main method for diagnosis in diarrhoea patients in Sweden and Cambodia.

In Paper III, human samples were cultured at NIPH in Cambodia. In addition, 200 randomly selected duplicate samples were cultured at the Clinical Microbiology Laboratory at Uppsala University (UU), for confirmation of the negative results obtained at NIPH using another selective media and another method of creating the microaerobic atmosphere, as described in Paper III. Limited human resources for bacterial analysis at NaVRI in Cambodia prevented culture analysis on the livestock samples. Thus, all livestock samples were cultured at SLU using ISO 10272:2006, the method recommended by the International Organization for Standardization (ISO). Standard protocols for culturing routinely used by the analytical laboratories were applied to livestock and human samples to enable assessment of the routine methods on samples collected under field conditions and frozen before being analysed. This involved using enrichment for livestock samples, but not for human samples.

To differentiate isolated *C. jejuni* from *C. coli*, the hippurate hydrolysis test was used. Isolates with positive hippurate enzyme activity were identified as *C. jejuni* and those with negative activity were further analysed by MALDI-TOF. The hippurate test is the only biochemical test available for species identification, but is often reported to show false negative results (*e.g. C. jejuni* fails to hydrolyse hippurate) (Al Amri *et al.*, 2007) (Rautelin *et al.*, 1999). MALDI-TOF was thus applied to confirm the hippurate negative isolates.

3.5.2 Conventional PCR

In contrast to detecting live bacteria that can grow, genome-based detection methods, such as PCR, detect DNA from both live and dead bacteria (Humphrey *et al.*, 2007). Real-time PCR detects multiplication of DNA fragments, whereas conventional PCR is directed to detect short fragments of the genome that may be multiplied and visualised following electrophoresis (Humphrey *et al.*, 2007).

Conventional PCR and gel electrophoresis was used to detect *Campylobacter* in human and livestock samples in Papers III and IV. Extraction of DNA in livestock samples was carried out at SLU, while DNA extraction from human samples and all PCR analyses were performed at UU using previously described primers specific for *C. jejuni* (targeting the *mapA* gene) and *C. coli*. (targeting the *ceuE* gene) (Denis *et al.*, 1999; Gonzalez *et al.*, 1997). The selection of PCR assay was based on previously used methods at UU and the species *C. jejuni* and *C. coli* were chosen as they represent the two most frequently isolated *Campylobacter* species in human campylobacteriosis patients (EFSA, 2015; Coker *et al.*, 2002). The PCR method demanded specific primers and experienced technical staff to prevent cross-contamination, neither of which was available at NIPH or NaVRI. In line

with recommendations from the Cambodian project partners, all analyses were carried out in Sweden, with human and livestock samples processed in the same laboratory at UU for consistency.



Figure 12. Influenza sampling on FTA[®] card.

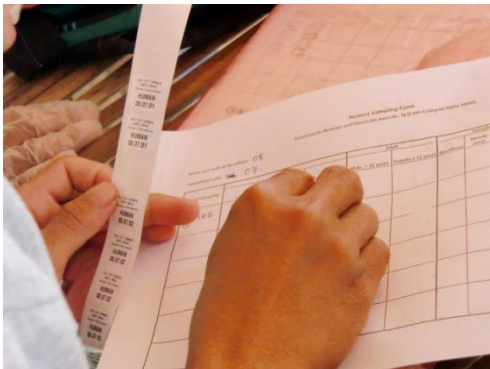


Figure 13. Human sampling form used during fieldwork.

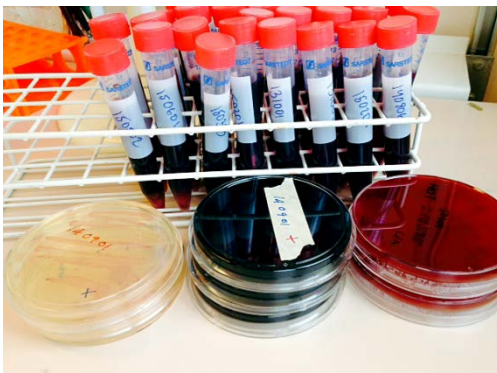


Figure 14. *Campylobacter* culture material. Vials with enrichment media at the back and Campyfood, mCCDa and blood agar plates at the front.

3.6 Spatial mapping and cluster scanning

Geographical position at the central point of the villages included in Papers I-IV was recorded using a handheld global positioning system (Garmin eTrex H). QGIS 2.0.1 software was used to produce maps on the distribution of villages with open source base map layers obtained from Open development Cambodia (opendevelopmentcambodia.net) and © OpenStreetMap contributors (openstreetmap.org).

Space-time scan statistics were used to enable detection of any spatial clusters of human *Campylobacter* positivity. SaTScan™ software, version 9.4.2, applying the Kulldorf method of retrospective permutation and the Bernoulli spatial model, was used to detect clusters of human *Campylobacter* positivity by village (Kulldorff *et al.*, 2005). The distribution and statistical significance of the clusters were explored by means of Monte Carlo replication. However, only non-significant spatial clusters were identified.

To further investigate clustering of *Campylobacter* positivity, the intra-cluster correlation coefficient (ICC) for human and livestock samples detected with *C. jejuni* and *C. coli* was calculated in Paper IV. Correlations between two observations in the same household or village were estimated by building unconditional logistic models, extracting the village and household level variances and assuming that the person/livestock-level variance was 3.29, as previously described (O'Connell *et al.*, 2008; Vigre *et al.*, 2004).

3.7 Statistical analyses

Statistical analysis in Papers I, III and IV was performed using SAS for Windows 9.3 (SAS Institute Inc., Cary, NC). Descriptive statistics were calculated in Paper I to define demographic characteristics, livestock management and the wealth of participating households. Rather than using monetary measures of wealth, which often vary between seasons (Howe *et al.*, 2008), an asset-based approach was applied to measure the household's socio-economic position (SEP). The SEP was defined by a wealth index, based on eight self-reported household belongings, multiplied by a weighting factor of 1-2 (Table 5). The method of adding up the sum of basically weighted belongings, instead of principal component analysis, was chosen due to the small number of indicators (eight household belongings) with a complete record from all 300 households. The SEP was further analysed by one-way analysis of variance (ANOVA) to test for differences in mean household wealth index between Kampong Cham, Battambang and Kampot provinces.

Table 5. Self-reported household belongings and weighting factors used to calculate the household wealth index

Household belonging	Weighting factor
All farming land owned by the household	1
House construction - concrete or brick	2
Roof construction - tiled	2
Safe water as main water source ¹	2
TV in the household	1
Mobile phone in the household	1
Vehicle or machine owned by the household ²	1
Cattle or buffalo owned by the household	1

¹Safe sources defined as bottled water, and boiled or filtered water from well, pond, stream or rainwater

²Bicycle, motorcycle, car, hand tractor, ox cart, rice miller or pumping machine

Differences in the proportion of *Campylobacter* positivity [Papers III and IV] and human gastrointestinal symptoms [Paper IV] were analysed with Pearson's chi-square, or Fisher's exact test when there were less than five observations per group. Multilevel logistic models were run to identify associations between household practices and various explanatory factors [Paper I] and to explore risk factors associated with *C. jejuni* and *C. coli* positivity in humans [Paper IV]. Since observations from the same household or village were assumed to be more alike than those from other households or villages, the effect of clustering was adjusted for. In Paper I, generalised estimating equations (GEE) were used to account for possible clustering of household practices within the same village (the focus in this paper was on population-averaged effects) (Hu *et al.*, 1998). One model was built for each of the household practices, with the practices as interchanging response variables against all the explanatory household factors: agro-ecological region; socio-economic position; number of people in the household; whether there were children in the household; number of chickens, ducks, other avian species, pigs, cattle and buffalo; whether the respondent knew of any zoonotic diseases; and whether the respondent perceived a likelihood of zoonoses in the village. In Paper IV, generalised linear mixed models were used with three levels of nested factors (person, household and village) as shown in Figure 15 (the focus in this paper was on subject-specific effects) (Hu *et al.*, 1998). Human *C. jejuni* or *C. coli* positivity was used as the response variable and univariable models were run with one of the 11 self-reported household practices (Table 4) as the explanatory variable. Furthermore were multivariable models run with either of the four groups of explanatory variables: the self-reported gastrointestinal symptoms in sampled

humans; number of chickens, ducks, pigs and cattle reared in the household; *C. jejuni*- or *C. coli*-positive samples from chickens, ducks, pigs or cattle; and number of days per month that poultry, pork and beef were consumed by the household.

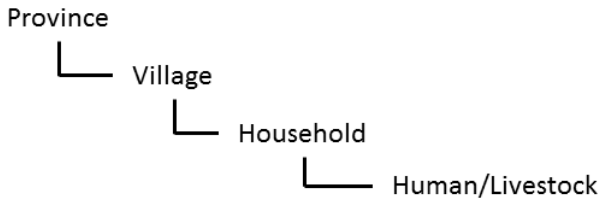


Figure 15. Hierarchical structure of data considered in Paper IV.

In addition, in order to explore associations between safe/unsafe water sources, human gastrointestinal symptoms and human *C. jejuni* or *C. coli* positivity, comparable multivariable models to those used in Paper IV were run with either gastrointestinal symptoms or human *C. jejuni/C. coli* positivity as the response variable against the explanatory variable of whether the household used safe water (bottled water, boiled/filtered water from a well, pond, stream or rainwater) as the main water source.

Multivariable models could not be run with the household practices as explanatory variables due to multicollinearity. Therefore the pattern of relationships among practices was examined and one factor was extracted for each group of practices to investigate whether people from households involved in more ‘hazardous practices’ had a higher probability of *Campylobacter* positivity than individuals from households involved in more ‘protective practices’. A principal factor method was used to extract factors with a promax (oblique) rotation. Two meaningful factors remained in the scree test (keeping eigenvalues greater than 1) and were retained for rotation. A practice was considered to load on a factor if the factor loading was greater than 0.3 (using a low cut-off) and the practices of daily collection of manure and capture and slaughter of wild animals were thus removed from the model (O'Rourke, 2013). In the final model, four practices loaded on the first factor (protective practices) and five practices on the second factor (hazardous practices), as shown in Figure 16.

Weighted estimated factor scores were assigned to each sampled person using proc score for the two factors. Generalised linear mixed models were run with *C. jejuni*- and *C. coli*-positive human samples as the response variable and the factor scores of factor 1 (protective practices) and factor 2 (hazardous

practices) as the explanatory variables. All factor-related models were non-significant, although practices were shown to be highly correlated.

While factor analysis is widely used on binary data, it is not fully justified for discrete data (Howe *et al.*, 2008). The results from factor analysis presented in this thesis should thus be interpreted with some caution.

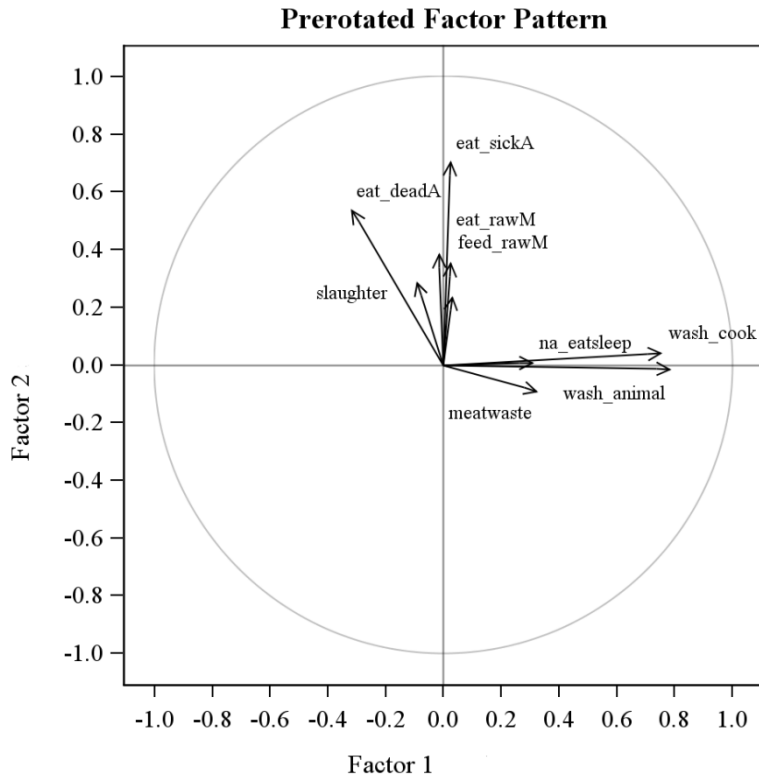


Figure 16. Loadings of protective factors (Factor 1) and hazardous factors (Factor 2) using a principal factor method.

4 Main results and discussion

4.1 Description of households and livestock management

A household was defined as a group of people making common arrangements for food and shelter. The median household size in the 300 households investigated was 5.0 (range 1-17), with a mean of 5.7 (Standard Deviation (SD) 2.1). The mean wealth index differed significantly between the three regions, with households in Kampong Cham province having the highest mean and households in Kampot province showing the widest range (Figure 17).

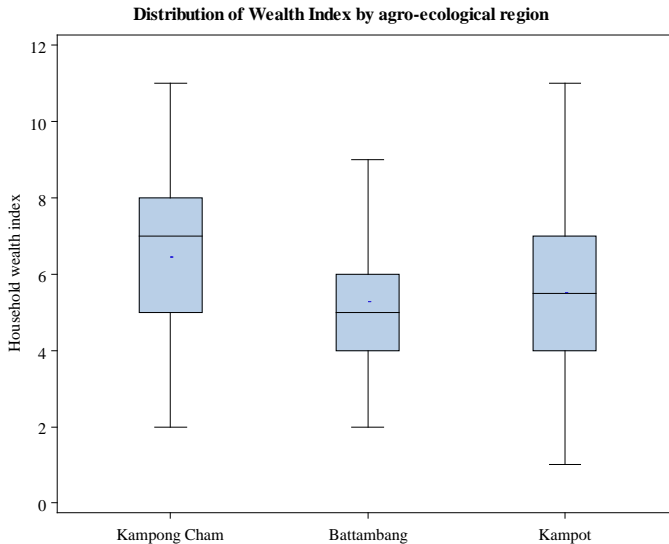


Figure 17. Boxplot showing household wealth index in 300 households in three different agro-ecological regions of Cambodia: Kampong Cham province (n=100), Battambang province (n=100) and Kampot province (n=100).

The most commonly occurring communicable diseases in the villages, as defined and reported by the respondents in the village questionnaire, were (in descending order) for livestock: Newcastle disease, gastrointestinal diseases, foot and mouth disease, fowl cholera and PRRS; and for humans: gastrointestinal disease, cold/flu, dengue fever, tuberculosis and typhoid fever. In the event of livestock falling ill, 16% of the households reported culling and consuming sick livestock and 17% reported selling both healthy and sick livestock. The preferred mode of sale was through an aggregator (middleman) or market vendor.

In the 300 households surveyed, chickens were the far most common species reared, followed by cattle, pigs, ducks and water buffalo. The majority of poultry (chickens, ducks and geese) and large ruminants (cattle and water buffalo) were reared in a free range system, whereas pigs were primarily confined. The responsibility for poultry, pigs and ruminants was shared between women, men and children in about 40% of the households. In the remaining households, women took more responsibility for poultry and pigs and men for ruminants. Poultry were reared mostly for income and family consumption, pigs and ruminants mostly for income only.

Understanding and considering the characteristics of rural livestock management, such as the purpose and gender roles, is important when discussing risk mitigation and preventive measures for zoonotic diseases. Knowledge on women's contributions to livestock keeping and the opportunities that livestock-related interventions could offer them is scarce (Kristjanson *et al.*, 2014). Moreover, the intra-household decision making, frequently with an uneven distribution between women and men, is rarely reflected upon (Hickler, 2007). Attention to gender dynamics should be a primary consideration in the development of zoonosis management programmes to achieve successful implementation. Interventions may also be directed towards certain target groups based on the livestock species involved and their contribution to livelihoods.

4.1.1 Zoonosis awareness and risk perception

In 69% of the households surveyed, at least one disease transmissible between animals and humans was known. Avian influenza was known by 65% of the households, while swine influenza, diarrhoea, tuberculosis and rabies were each known by less than 5%. Only 6% of the households regarded disease transmission between livestock, humans and wildlife as likely in the village.

The high awareness of avian influenza can possibly be explained by nationwide influenza awareness activities carried out in Cambodia since the first

outbreaks of HP H5N1 in 2004. It was remarkable, however, that few of the households surveyed reported perceiving a threat of zoonoses.

4.2 Household practices associated with zoonosis exposure [Paper I]

Associations between household practices (see Table 4) and potential explanatory household factors and confounders were analysed in separate models. Models with significant associations are presented in Table 6. Several household practices were associated with more people and livestock in the household and most practices were associated with the region, a finding possibly explained by diverse farming opportunities deriving from different climate and physical conditions in the three regions. Regional associations may also depend upon differences in socio-economic opportunities and development support. One explanation for the higher average wealth index in the Kampong Cham households might be that those villages had more than twice as many development projects ongoing than villages in the other two provinces.

A positive effect of zoonosis knowledge was associated with the practices of washing hands before and after cooking and after handling live animals, yet a contrasting association was found between zoonosis knowledge and the practices of home slaughtering and feeding animals uncooked slaughter waste. These hazardous practices were more frequently reported in households where the respondent knew of a zoonosis, and feeding animals uncooked slaughter waste was also more frequently reported in households where the respondent perceived a likelihood of zoonosis transmission in the village. The knowledge-to-action gap identified should be seen in the light of influenza information campaigns regularly run in Cambodia since 2004. Several studies have reported raised awareness among the rural population of human-animal disease transmission since these public awareness campaigns started (Khun *et al.*, 2012; Hickler, 2007; Ly *et al.*, 2007), but despite this, practices associated with zoonosis transmission persist and messages provided on disease control seem to penetrate only partly down to the level of farm practices.

The socio-economic position of households has been shown by others to influence precautionary household practices, as better economic conditions allow upgrading of housing, sanitation and purchase of hygiene products (Rabbi & Dey, 2013; Nasinyama *et al.*, 2000). Such associations were not found in Paper I. Instead, the practice of chasing animals away from sleeping and food preparation areas was associated with lower wealth index. A possible

Table 6. Associations between the response variable household practice and the explanatory household factors: agro-ecological region, socio-economic position, number of people, number and species of livestock reared, and zoonosis awareness (n=300)

Household practice ¹	Explanatory factors ¹	OR (95% CI)	P-value
Feed animals uncooked slaughter waste	Region		0.0009
	KPC vs KT	9.6 (3.5-26)	<0.0001
	KPC vs BB	12 (4.5-32)	<0.0001
	Knowledge of zoonoses	2.2 (1.0-4.5)	0.04
	Perceived likelihood of zoonoses	7.5 (2.2-26)	0.001
Eat animals found dead	Region		0.04
	KPC vs KT	ns	ns
	KPC vs BB	0.4 (0.2-0.7)	0.003
	Buffalo ²	1.3 (0.9-1.7)	0.14
Wash hands with soap before and after cooking	Region		0.02
	KPC vs KT	ns	ns
	KPC vs BB	ns	ns
	Knowledge of zoonoses	1.4 (1.3-7.3)	0.01
Wash hands with soap after handling live animals	Region		0.02
	KPC vs KT	ns	ns
	KPC vs BB	ns	ns
	Knowledge of zoonoses	1.4 (1.3-7.3)	0.01
Keep live animals away from sleeping and food preparation areas	Region		0.0002
	KPC vs KT	67 (9.5-470)	<0.0001
	KPC vs BB	2.9 (1.5-5.5)	0.0013
	Wealth score	0.8 (0.7-0.9)	0.0003
Bury or burn meat waste products	Household size	1.3 (1.1-1.5)	0.01
Daily collection of manure in-and outdoor	Cattle	1.2 (1.0-1.5)	0.01
Slaughter domestic animals	Region		0.0007
	KPC vs KT	0.2 (0.1-0.3)	<0.0001
	KPC vs BB	ns	ns
	Household size	1.5 (1.1-1.3)	0.002
	Chicken	1.0 (1.0-1.1)	0.004
	Knowledge of zoonoses	1.9 (1.1-3.2)	0.02

¹Only response variables and explanatory factors with significant ($P < 0.05$) associations from the modelling are shown.

²Buffalo retained in the model despite a non-significant p-value, as removal caused a change in the province estimate of more than 20%, suggesting that number of buffalo was a confounder in the model.

Kampong Cham province (KPC), Kampot province (KT), Battambang province (BB). Odds ratio (OR).

explanation could be that animals can more easily enter cooking and sleeping areas in poor households with an open housing construction. Households with a lower wealth index will thus actively have to chase away animals, while in the wealthier households the more solid housing construction used will keep animals out. Interestingly, when the data were analysed at the regional level, the socio-economic position of households was not associated with household practices. Households in Kampong Cham province had the highest average wealth index, but precautionary household practices were not reported more frequently there than in the other two regions.

4.3 Influenza A virus in pigs and poultry [Paper II]

Of the 270 households in where influenza samples were obtained, 260 (96%) reared chickens, 104 (39%) ducks, 159 (59%) pigs and 3 (1%) pigeons. A total of 1301 samples were screened for IAV, including nasal swabs from pigs, tracheal and cloacal swabs from chickens and ducks, and swabs with faecal material from pigeons. Influenza A virus was detected in swabs from 10 animals (1.3%) originating from all regions sampled, as shown in Table 7 and Figure 18.

4.3.1 Swine influenza virus

Swine influenza virus was detected in three samples (1.5%), and classified as H3N2 by sequence analysis of the HA and NA genes. The HA gene of the three SIVs shared 89-100% identity at the nucleotide level and clustered in two different phylogenetic groups. In Kampong Cham province, one SIV was detected. It was closely related to that in novel swine H3N2 viruses, represented by A/Swine/BinhDuong/03-06/2010 (Ngo *et al.*, 2012), a TR H3N2 virus with HA and NA genes related to 2004-2006 seasonal human H3N2 viruses reported in Vietnam in 2010. In Battambang province, two SIVs were detected. They were derived from other TR H3N2 viruses and formed a separate subclade together with A/swine/Hanoi/415/2013 (Baudon *et al.*, 2015) within the American TR H3N2 swine viruses. The NA genes shared 99% identity at the nucleotide level and were all closely related to A/swine/Hanoi/415/2013. No SIV was detected in samples collected in Kampot province in 2013.

The TR H3N2 detected among pigs in Paper II has not previously been reported in Cambodia, but antibodies in pigs to human H3N2 have been described (Netrabukkana *et al.*, 2014; Rith *et al.*, 2013). Interestingly, the TR H3N2 viruses from Kampong Cham and Battambang provinces were derived

from two distinct subclades, showing that several TR lineages are present in Cambodian pigs.

Emergence of resistant IAV subtypes poses a worldwide threat in influenza treatment and has been described in different hosts, including humans, pigs and poultry (Dong *et al.*, 2015). The profiles of the SIV strains identified in Paper II suggest susceptibility to the neuraminidase inhibitors oseltamivir and zanamivir (Gubareva, 2004), but resistance to amantadine (Dong *et al.*, 2015; Belshe *et al.*, 1988). These results support findings on human IAV susceptibility in Cambodia showing 100% amantadine-resistance, whereas sensitivity to oseltamivir and zanamivir remains high (Dong *et al.*, 2015; Horm *et al.*, 2014; Fourment *et al.*, 2010). Cambodia's resistance profile is suggested to reflect a strong influence from neighbouring countries, linked to drug-use pressure and fitness mutations of IAV (Horm *et al.*, 2014), but the role of livestock in the emerging IAV resistance remains unclear.

Table 7. Cloacal (C), nasal (N) and tracheal (T) samples testing positive for influenza A virus (n=751)

Province	Village	Sampling year	Livestock type	Sample type	Influenza subtype	Accession number ²
Kampong Cham	Pror Sam	2011	Chicken	C	H3N8	EPI702901- EPI702908
Kampong Cham	Pror Sam	2011	Chicken	T	H4N6	EPI702959- EPI702966
Kampong Cham	Pror Yuk	2011	Chicken	C, T	H6N2	EPI702264- EPI702271
Kampong Cham	Tang Krang	2011	Pig	N	H3N2	EPI702169- EPI702176
Battambang	Kandal Tbong	2012	Chicken	T	H3N6	EPI702676- EPI702683
Battambang	Chranieng	2012	Pig	N	H3N2	EPI702185- EPI702192
Battambang	Chranieng	2012	Pig	N	H3N2	EPI702177- EPI702184
Kampot	Ta Did	2013	Chicken	T	H6N2	EPI702609- EPI702616
Kampot	Ta Did	2013	Chicken	C, T	Influenza A ¹	-
Kampot	Beong Tapream	2013	Duck	C	H6N8	EPI702347- EPI702354

¹Subtype could not be confirmed.

²Nucleotide sequences accession number obtained in the Global Initiative on Sharing All Influenza Data.

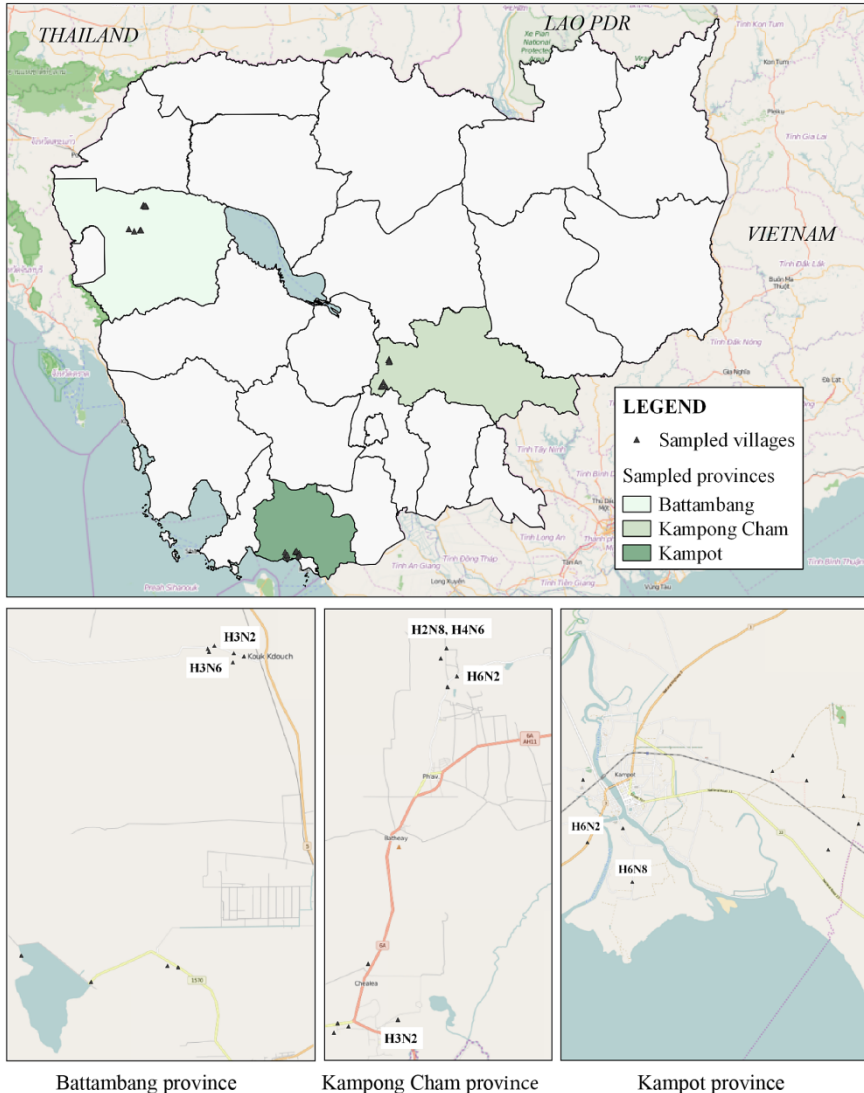


Figure 18. Map of Cambodia showing the geographical distribution of villages sampled, including sites with positive influenza samples. Open Development Cambodia (www.opendevdevelopmentcambodia.net) and © OpenStreetMap contributors (openstreetmap.org).

4.3.2 Avian influenza virus

Low Pathogenic Avian Influenza viruses of various subtypes were found in seven poultry samples and analysed by sequence analysis. In one of the matrix rRT-PCR positive AIV samples no sequences were obtained, possibly due to a sensitivity difference between the assay used for screening (matrix rRT-PCR) and the one used for genome sequencing. The sample was, however, negative

for H5, H7, H9 or N1 gene specific rRT-PCR. In the remaining six positive samples, phylogenetic analysis of the HA gene of the respective subtype showed that all LPAI viruses were similar to AIVs previously isolated in Southeast Asia. The BLAST search results and phylogenetic analysis of the internal genes suggested that the different subtypes detected have various genetic components as a result of frequent reassortment and exchange of internal viral proteins between multiple influenza subtypes in poultry and wild birds in the region, including H9N2 and HP H5N1.

The overall prevalence (1.3%) of AIV detected in Paper II was considerably lower than the 6% and 14% H5N1 positivity found in pooled faeces samples from poultry in Cambodian live bird markets in 2011 (Horm *et al.*, 2013) and 2013 (Buchy, 2014), respectively. The lower positivity and absence of HP AIV compared with those market studies is most likely due to several factors. In this thesis work individual tracheal and cloacal swab samples were collected, whereas pooled samples, primarily from ducks, were analysed in the market studies. Moreover, of the households included here, 17% reported selling their livestock if disease was detected within the flock, most commonly through an aggregator or market vendor. Consequently, infected poultry accumulate in live bird markets, where a large number of birds from different sources aggregate. If AIV is introduced, infection is easily maintained and virus reassortment is facilitated through the mixture of domestic and wild bird species, including species able to maintain silent infection (Pfeiffer *et al.*, 2013).

4.3.3 Transboundary and methodological aspects

Trade in domestic poultry occurs frequently across borders between Cambodia and neighbouring countries and is likely to explain co-circulation of AIV lineages in the Mekong sub-region (Sorn *et al.*, 2013; Chen *et al.*, 2006). Wild migratory birds may serve as an additional source, but continued reintroduction of virus seem less common given the establishment of AIV, in particular HP H5N1 lineages (Chen *et al.*, 2006). Clustering of gene segments identified in Paper II supports the idea of transboundary AIV and SIV circulation in Cambodia, most likely driven by trade in livestock. In recent years Cambodia has imposed sporadic importation bans on pigs and poultry, mainly from Vietnam due to concerns about disease incursions, but consumer demands and established trading networks seem to sustain informal cross-border trading (Sorn *et al.*, 2013; Sieng *et al.*, 2012; Van Kerkhove *et al.*, 2009).

Parallel collection of tracheal and cloacal samples in poultry proved valuable, considering that AIV was detected in both types of samples in only two out of seven poultry confirmed positive. In addition, sampling on FTA[®] cards, which has previously been reported to permit sensitive diagnosis of

influenza (Abdelwhab *et al.*, 2011), proved to be convenient and may have the potential to overcome some of the critical barriers to influenza surveillance, related to cold-chain transport and laboratory capacities, identified in Southeast Asia (Trevenne *et al.*, 2011).

4.4 *Campylobacter* in livestock and humans [Papers III and IV]

4.4.1 *Campylobacter* detection by routine culturing and multiplex PCR

Samples were collected and analysed from 853 livestock in 249 households and from 681 humans in 269 households. Both human and livestock samples were obtained and analysed from 229 households. Livestock samples from 40 households in Kampot province were unfortunately never analysed due to unintended omission of the samples from the final shipment.

Faecal samples from chickens, ducks, pigs, cattle, water buffalo, quail, pigeons and goose were tested by culture and PCR (Figure 19). Among the 853 livestock samples, *Campylobacter* were detected by culture in 106 samples (12%); 72 samples (68%) tested positive for *C. jejuni* and 31 samples (29%) for *C. coli*, whereas in three samples (3%) the *Campylobacter* species could not be determined. In the PCR analysis, *Campylobacter* were detected in 352 samples (41%). Among all the positive samples in PCR analyses, *C. jejuni* only were detected in 177 (50%), *C. coli* only in 124 (35%) and both *C. jejuni* and *C. coli* in 51 samples (14%). None of the human samples tested positive for *Campylobacter* by culture, but by PCR 82 samples (12%) were *Campylobacter* positive; *C. jejuni* were detected in 66 samples (80%) and *C. coli* in 16 samples (20%) (Figure 19).

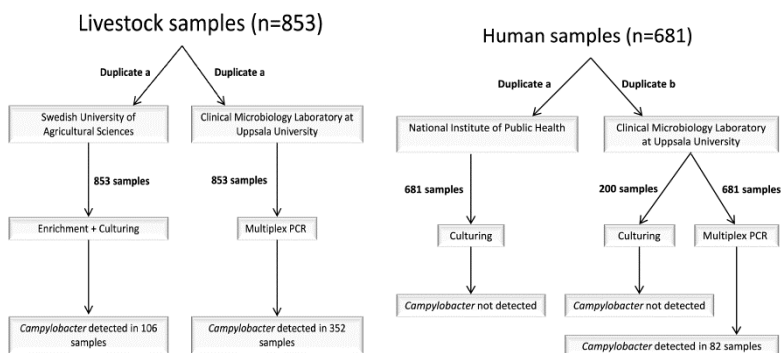


Figure 19. Analytical procedure and overall results for *Campylobacter* detection in livestock and human samples.

The substantially lower detection rate of *Campylobacter* by routine culture compared with PCR was most likely due to multiple factors, such as lower sensitivity of culture in general and non-optimal conditions for bacterial survival. It is important to note that PCR detects bacterial DNA and not only live bacteria, and hence a proportion of the non-culturable samples may contain dead bacteria or levels of viable *Campylobacter* below the detection limit. In settings where people are frequently *Campylobacter* infected, convalescent-phase samples may also remain positive by PCR while culture is negative (Havelaar *et al.*, 2009). Interestingly and in contrast to other studies (Al Amri *et al.*, 2007; Denis *et al.*, 1999; Linton *et al.*, 1997), the PCR assay applied in Papers III and IV was not 100% sensitive compared with culture in detecting *C. coli*/*C. jejuni*, as shown in Table 8. The failure of PCR to detect *C. jejuni* and/or *C. coli* in 15 samples that tested positive by culture was most likely due to insufficient faecal material remaining in the vial once the swab had been removed for culturing purposes.

Table 8. *Campylobacter* species detected by routine culture and multiplex PCR in faecal livestock samples (n=853)

Molecular detection by PCR	Detection by routine culture		
	<i>C. jejuni</i>	<i>C. coli</i>	Unspecified ¹
<i>C. jejuni</i>	50	5	0
<i>C. coli</i>	3	14	2
Mixed infection (<i>C. jejuni</i> and <i>C. coli</i>)	8	9	0
No detection of <i>C. coli</i> or <i>C. jejuni</i>	11	3	1

¹Confirmed *Campylobacter* spp., no species could be determined.

In contrast to the livestock samples, none of the human samples tested positive for *Campylobacter* by culture, despite duplicate analysis of 200 samples in two unlike laboratories applying the protocols routinely used by the laboratories in *Campylobacter* diagnosis. These differences are most likely due to differences in handling and culturing procedures. The livestock faecal samples were immediately placed in vials with transport medium, whereas the self-collected human samples were stored for up to 6 hours before faecal material was transferred into the vials. Additionally, the standard protocols included enrichment for livestock, but not for human samples. However, in a final attempt to recover *Campylobacter* in 20 human samples that tested positive by PCR using the same enrichment step as for livestock samples, the human samples remained negative for *Campylobacter*.

In the rural field conditions, timely culture was not feasible. Others (Platts-Mills *et al.*, 2014; Bullman *et al.*, 2012) have previously reported failed growth

of *Campylobacter* in samples cultured with a 24-h delay. The one-day time span from sampling to freezing in Papers III and IV most likely reduced the number of viable and cultivable bacteria. In addition, despite employing a medium successfully used by the research group at UU for long-term preservation of *Campylobacter*, storage of samples at -70 °C may have caused further damage, as *Campylobacter* cells exposed to freezing have previously been shown to be less able to recover (Wasfy *et al.*, 1995).

4.4.2 *Campylobacter* prevalence in livestock and humans

Campylobacter jejuni, *C. coli* or both were detected by PCR in 56% of the chickens, 24% of the ducks, 72% of the pigs and 5% of the cattle tested (Table 9). None of the samples collected from water buffalo (n=25) and quail (n=1) tested positive for *Campylobacter* by PCR, but one of the pigeon samples (n=3) and the only goose sample (n=1) tested positive for *C. jejuni*. In chickens and ducks, *C. jejuni* was the most prevalent *Campylobacter* species, while in pigs *C. coli* was the most prevalent. The percentage of sampled households with at least one positive sample by PCR was 65% for chickens, 25% for ducks, 78% for pigs and 6% for cattle. Clustering of positive livestock samples was weak within households (ICC=0.05 (variance estimate 0.17)) and non-detectable within villages.

Table 9. Detection of *Campylobacter* by routine culture and multiplex PCR in faecal samples from chickens, ducks, pigs and cattle

Method	Number (%) of samples testing positive			
	Chickens (n=353)	Ducks (n=101)	Pigs (n=162)	Cattle (n=207)
Culture: positive for <i>Campylobacter</i>	87 (25)	5 (5)	11 (7)	2 (1)
<i>C. jejuni</i>	66 (19)	4 (4)	0	1 (0.5)
<i>C. coli</i>	19 (5)	1 (1)	10 (6)	1 (0.5)
<i>C. spp.</i> ¹	2 (1)	0	1 (1)	0
PCR: positive for <i>C. jejuni</i> , <i>C. coli</i> or both ²	198 (56)	24 (24)	117 (72)	11 (5)
<i>C. jejuni</i>	185 (52)	18 (19)	19 (12)	4 (2)
<i>C. coli</i>	50 (14)	8 (8)	110 (68)	7 (35)

¹Confirmed *Campylobacter* spp., no species could be determined.

²51 samples tested positive for both *C. jejuni* and *C. coli*.

In humans, *C. jejuni* were more prevalent than *C. coli* and one or the other were detected in 19% of children below 16 years and in 8% of adults by PCR (Table 10). The prevalence was significantly higher in children than in adults ($P<0.001$), but no significant difference in the proportion of positive samples

could be determined between the three age groups <2 years, 2-5 years and 6-15 years. At least one positive human sample was detected in 66 households (24%), with quite strong clustering of positive samples within households (ICC=0.14 (variance estimate 0.47)) and weaker clustering within villages (ICC=0.02 (variance estimate 0.07)).

The livestock and human prevalence observed was similar to that detected by culture in India (Gupta *et al.*, 1991), Thailand (Bodhidatta *et al.*, 2010) and Vietnam (Carrique-Mas *et al.*, 2014). Interestingly, the prevalence of *C. jejuni* and *C. coli* in chickens was considerably lower than the *Campylobacter* positivity reported in poultry meat sold in wet markets in Cambodia (81%, by culture) (Lay *et al.*, 2011) or in poultry meat in rural Thai markets (80%, by culture) (Bodhidatta *et al.*, 2013). Although a different assay (PCR) was used in Papers III and IV, the results suggest a higher rate of *Campylobacter* positivity on chicken meat than in chicken faeces, likely caused by cross-contamination during slaughtering and meat handling. In Paper IV, unlike in other studies (Coker *et al.*, 2002; Rao *et al.*, 2001), there was no significant difference in *Campylobacter* positivity between the different age groups of children. One possible explanation could be the previously discussed high sensitivity of PCR detecting low numbers of *Campylobacter* in comparison with culture. Finally, negligible differences were found in *Campylobacter* prevalence between the three regions, which were sampled in different seasons, suggesting a non-regional or seasonal preference of *Campylobacter* positivity.

Table 10. *Detection of C. jejuni and C. coli by multiplex PCR in faecal samples from children and adults*

Method	Number (%) of samples positive			
	Child < 2 years (n=34)	Child 2-5 years (n=53)	Child 6-15 years (n=185)	Adult >15 years (n=409)
PCR positive for <i>C. jejuni</i> or <i>C. coli</i>	8 (24)	7 (13)	36 (19)	31 (8)
PCR positive for <i>C. jejuni</i>	5 (15)	7 (13)	30 (16)	24 (6)
PCR positive for <i>C. coli</i>	3 (9)	0	6 (3)	7 (2)

4.4.3 Self-reported symptoms of gastrointestinal disease

Symptoms of abdominal pain, diarrhoea, fever and vomiting during the two-week period preceding the sampling, as defined by the respondent or, for younger children, by the parent, were recorded for each person sampled (Table 11). Fever was the most commonly reported symptom (21%), but showed no statistically significant difference between age groups. Abdominal pain was the second most commonly reported symptom (14%) and diarrhoea the third

(12%). Diarrhoea was more frequently reported in children below 6 years than in adults and children 6-15 years of age ($P<0.001$).

The symptoms were self-reported and based on personal perception rather than set case definition. The perception method, which is often applied in cross-sectional interviews, has some innate weaknesses such as variability in the definition of symptoms, inaccurate reports of illness and recall error. However, case definitions could have introduced confusion in the present analysis, as four different symptoms were reported, and therefore personal perception was used. Others have also suggested a reduction in recall bias using perception definitions compared with case definitions when a recall period of two weeks or longer is applied (Goldman *et al.*, 1998; Baqui *et al.*, 1991).

Table 11. Rate of self-reported (or reported by parent for younger children) gastrointestinal symptoms during the two-week period prior to sampling (n=681)

Sample type	Abdominal pain Number (%)	Diarrhoea Number (%)	Fever Number (%)	Vomiting Number (%)
Child <2 years (n=34)	2 (6)	11 (32)	9 (26)	1 (<1)
Child 2-5 years (n=53)	7 (13)	13 (25)	16 (30)	2 (4)
Child 6-15 years (n=185)	22 (12)	15 (8)	39 (21)	2 (1)
Adult >15 years (n=409)	62 (15)	43 (11)	79 (19)	4 (1)

4.4.4 Risk factors associated with *Campylobacter* positivity

Potential risk factors associated with human *C. jejuni* or *C. coli* positivity were identified in the univariable and multivariable models using data from the 269 households where human samples were obtained. No associations were found between the outcome variable *C. jejuni* or *C. coli* in human samples and the self-reported gastrointestinal symptoms; the number of chickens, ducks, pigs or cattle reared; or detection of *C. jejuni* or *C. coli* among the household's chickens, ducks, pigs or cattle. The household practices of home slaughtering, allowing animals into sleeping and food preparation areas, and eating undercooked meat were associated with increased odds of human *C. jejuni* positivity, whereas frequent consumption of beef was associated with decreased odds (Table 12). The probability of *C. jejuni*-positive samples was higher in the subset models of children below 16 years of age for the household practice of home slaughtering. None of the other household practices (see Table 4) was associated with *C. jejuni* or *C. coli* in samples from children. Detection of *C. coli* was associated with frequent consumption of poultry, both when all the human samples were included in the model and when the child

subset model was used. Frequent consumption of pork was associated with detection of both *C. jejuni* and *C. coli* in the child model (OR 1.1, $P=0.04$). All models with significant associations between *C. jejuni* or *C. coli* detected in human samples and explanatory variables are presented in Table 12.

Absence of associations between *Campylobacter* detection and gastrointestinal symptoms has been reported previously in low-income countries (Randremanana *et al.*, 2012; da Silva Quetz *et al.*, 2010), and is most likely due to the development of protective immunity, as frequent exposure to *Campylobacter* at a young age may boost the immune response to protect against clinical disease, but not necessarily against transient positivity (Havelaar *et al.*, 2009). Regardless of symptoms, however, *Campylobacter* positivity is important in rural low-income areas, particularly in the case of children, and some studies have found asymptomatic *Campylobacter* infection to be associated with malnutrition and reduced growth (Lee *et al.*, 2013; da Silva Quetz *et al.*, 2010).

Table 12. Significant associations in generalised linear mixed models between the outcome variable detection of *Campylobacter jejuni* or *C. coli* by PCR in human samples ($n=681$) and samples from children only ($n=272$), and explanatory variables measured at household level

Outcome variable	Explanatory variable	OR (95% CI)	P-value
<i>C. jejuni</i> detected in human sample	Slaughter domestic animals	2.4 (1.2-4.8)	0.01
	Allow animals in sleeping and food preparation areas	2.8 (1.2-6.5)	0.02
	Eat undercooked meat	6.6 (1.0-44)	0.05
	Number of days per month that beef is consumed	0.9 (0.7-1.0)	0.05
<i>C. coli</i> detected in human sample	Number of days per month that poultry is consumed	1.2 (1.1-1.3)	0.006
<i>C. jejuni</i> detected in sample from child <16 years	Slaughter domestic animals	4.9 (1.7-14)	0.004
<i>C. coli</i> detected in sample from child <16 years	Number of days per month that poultry is consumed	1.2 (1.0-1.4)	0.02

Odds ratio (OR). Confidence Interval (CI).

In high-income countries, the majority of human campylobacteriosis cases seem to be related to chicken, including indirect transmission and not only the usual eating or handling routes (EFSA, 2015; WHO, 2013; Nichols *et al.*, 2012). The effect of poultry rearing could not be investigated in this thesis, however, as nearly all households kept poultry. Livestock keeping *per se* was not associated with an increased probability of human *Campylobacter* positivity, even when *Campylobacter* were detected in the livestock reared.

Instead, the biosecurity measures and hygiene precautions applied within the household seemed more important and household risk factors were detected throughout the meat production chain, from free-ranging livestock and home slaughtering, to unsafe meat preparation and consumption. Such results suggest that future actions targeting the entire meat production chain are needed to reduce the burden of human *Campylobacter* infection. Moreover, as reported in Paper I, livestock are mainly produced to generate an income and often sold by households. Efficient *Campylobacter* control ought thus to move beyond the household level to improvements in hygiene practices targeting external actors along the meat production chain, such as middlemen, abattoirs and consumers.

Consumption of poultry was associated with human *C. coli* positivity, but not with *C. jejuni* positivity, which was remarkable since *C. jejuni* was detected in 45% of poultry samples and *C. coli* in only 13%. Nevertheless, some care is needed before generalising these results, as only 16 human samples tested positive for *C. coli*. Consumption of beef was found to be protective against human *C. jejuni*, although borderline significant, but an explanation for this remains unclear. The data did not support the theory that beef was more frequently consumed in affluent households, affording a higher hygiene standard, or that an increase in beef consumption decreased poultry and pork consumption. Finally, the high odds ratios presented for undercooked meat consumption should be interpreted with caution, as the association with *C. jejuni* positivity was only borderline significant, with a wide confidence interval (CI: 1.0-44).

In the additional models testing associations between safe/unsafe water sources, gastrointestinal symptoms and human *C. jejuni* or *C. coli* positivity, symptoms of abdominal pain (OR 2.8, $P=0.004$) and fever (OR 2.8, $P=0.008$) were associated with an unsafe water source, whereas diarrhoea, vomiting and human *C. jejuni/C. coli* positivity were not significantly associated with the water source.

The identified links between poor water quality and disease symptoms were expected and have been extensively described by others (Ashbolt, 2004; Prüss *et al.*, 2002). Safe drinking water is, in addition to improved sanitation and hygiene, crucial for improved health in rural communities in low-income countries, but data on the role of water as a carrier for zoonotic pathogens is scarce. The role of the environment in the zoonosis transmission pathway is in general poorly understood. Such data could further enhance our understanding of the zoonosis epidemiology.

5 Conclusions

This thesis provides new knowledge on human-livestock interactions and the presence of zoonoses (here influenza A virus and *Campylobacter*) in rural Cambodian households. The specific conclusions drawn from the results presented in this thesis are as follows:

- Household practices linked with zoonosis exposure were common, but few of the households surveyed reported the threat of zoonosis to be a concern in their village.
- Zoonosis awareness did not markedly reduce household practices associated with increased zoonosis exposure, indicating a knowledge-to-action gap among smallholders.
- Backyard farmed pigs and poultry had a low prevalence of influenza A virus, but virus reassortment involving potentially zoonotic and pandemic subtypes appeared frequent. Highly pathogenic subtypes were not found.
- Routine culture was insufficiently sensitive in detecting *Campylobacter* in frozen and thawed field samples, suggesting PCR as the preferred method when timely culture is not feasible.
- *Campylobacter*, as detected by PCR, was highly prevalent in faeces samples from children, poultry and pigs.
- Several household practices along the meat production chain, from livestock rearing to meat consumption, were associated with *Campylobacter* positivity in humans.

Taken together, these new findings on zoonosis epidemiology and household risk factors can help guide future interventions in zoonosis prevention, detection and control.

6 The development context and way forward

Throughout the work presented in this thesis, we have produced new information on the livestock-human interplay in rural Cambodian households. We have tested alternative sampling and diagnostic tools for field samples and detected influenza virus in livestock and *Campylobacter* in livestock and humans. Moreover, we have identified zoonotic risk factors associated with human *Campylobacter* positivity. Although we have gained insights into the epidemiology of influenza and *Campylobacter* it remains to transfer the new knowledge into efficient zoonosis management in Cambodian smallholdings.

Low-income countries often lack an evidence-base for planning and targeting zoonosis control efforts. This thesis provides some evidence that may assist in zoonosis control, such as a need: to reconsider the focus in public awareness campaigns; to continue with targeted AIV surveillance in live bird markets, but monitor influenza evolution also in backyard flocks; and to emphasise slaughter hygiene as a critical control point for *Campylobacter*. Implementation and the final decision on control measures should, however, be taken and led by the Cambodian authorities.

The work presented in this thesis was conducted within the framework of a multi-disciplinary project on zoonoses that had capacity building in zoonosis management as one of its key objectives. Veterinary public health and human health institutions worked in tandem throughout the project and Cambodian laboratory personnel from both disciplines were trained together in *Campylobacter* analysis. These strong veterinary-human health links added value and are important for efficient zoonosis management. Thus similar trans-disciplinary ‘One Health’ approaches should permeate future research and development initiatives on zoonoses.

This thesis focused on two very different zoonotic pathogens, in order to reflect the diversity of zoonoses. These were influenza A virus, epizootic and

comparatively well studied in Cambodia, and *Campylobacter*, regarded as endemic, but with very limited data available from Cambodia. The two pathogens were selected in consultation with the Cambodian project partners based on relevance and available expertise. We believe that the results in this thesis are applicable to a range of zoonotic diseases in tropical areas. However, it would be of great interest to study other pathogens of importance in Cambodia. Such research could further advance the current understanding of zoonosis epidemiology and control.

In Cambodia, many zoonoses are neglected, partly due to the absence of reliable qualitative and quantitative data. The neglect is further exacerbated by international priorities on diseases that pose an emerging global threat, but of comparatively limited importance to impoverished communities. Further research on neglected zoonoses and new tools to assess the zoonosis burden would be valuable and could provide an advocacy base to approach policy makers. Moreover, understanding and considering purpose and gender roles in livestock rearing, as done in this thesis, could assist in better targeted interventions in zoonosis management and serve as means of empowering marginalised groups.

The ability to detect and identify infection is crucial in zoonosis surveillance and control. Data collection and disease recording systems are often fragmentary in Cambodia and diagnosis is commonly based on clinical manifestations rather than positive tests results. It is imperative in such conditions to have accurate, easily used and robust diagnostic tools for field application. Influenza sampling on FTA[®] cards and *Campylobacter* detection by PCR worked well in this thesis and proved appropriate for surveillance in the prevailing rural field conditions. By exploring, exploiting and evaluating sampling and diagnostic techniques for field use, additional suitable techniques can be made available for zoonosis detection in tropical areas.

In summary, by applying a 'One Health' approach, this thesis presents novel data on the human-livestock interface, influenza and *Campylobacter* in Cambodian smallholdings. The results provide a foundation for further research and can help guide research and development institutions in the planning and implementation of studies and measures to manage zoonoses at the source, thereby improving health and livelihoods throughout rural tropical areas.

7 Populärvetenskaplig sammanfattning

De flesta infektionssjukdomar hos människor är zoonoser, nämligen sjukdomar som kan överföras mellan djur och människor. Varje år insjuknar runt en miljard människor i zoonoser, med miljontals dödsfall till följd. Fattiga hushåll i låginkomstländer anses vara särskilt hårt drabbade.

Kambodja är ett av världens högriskområden för uppkomsten av zoonoser och nya infektionssjukdomar. Merparten av Kambodjas invånare bor på landsbygden med odling och djurhållning som huvudsakliga inkomstkällor. Förutom att vara en viktig inkomstkälla är djuren av betydelse för tillgång på kött, mjölk och ägg, som dragdjur och för produktion av naturgödsel. I dessa småskaliga jordbruk lever djur och människor många gånger nära varandra och djuren saknar som regel inhägnader. Tillgången till sjukvård för människor och djur är ofta begränsad och hygienrutiner är sällan tillräckliga, vilket ökar risken för zoonoser.

Syftet med avhandlingen var att undersöka om och hur samspelet mellan djur och människor på Kambodjas landsbygd bidrar till att sprida zoonoser. Vi valde att studera förekomsten av två olika zoonotiska smittämnen som modell: influensa A-virus, som ger upphov till bland annat fågelinfluensa, och campylobacter-bakterier, som orsakar diarré och kräkningar hos människor, i synnerhet barn. Dessutom jämförde vi odling av campylobacter med polymeraskedjereaktion (PCR) på frusna och tinade träck- och avföringsprover samt identifierade riskfaktorer för zoonotisk campylobacter-smitta inom hushållen.

Totalt studerades 300 hushåll i tre olika provinser och varje hushåll intervjuades om hushållets sammansättning, levnadsstandard, hygienrutiner, djurhållning och kunskap om zoonoser. I anslutning till intervjuerna togs också avförings- och träckprover från människor och livsmedelsproducerande djur, i huvudsak ankor, duvor, grisar, kor, kycklingar och vattenbufflar, för

campylobacter-analys samt tryn-, kloak- och luftrörsprov från grisar och fjäderfä för influensaanalys.

Bland resultaten återfanns bybornas riskbeteenden, som att låta djuren gå fritt där man sov och åt, och äta sjuka och självdöda djur, vilka förekom i en fjärdedel av hushållen. Trots det ansåg endast sex procent av hushållen att det fanns någon risk för spridning av zoonoser mellan djur och människor i deras by. Hushållens kunskaper om zoonoser hade ringa betydelse för riskbeteenden.

Influensavirus påträffades hos lite mer än en procent av provtagna grisar och fjäderfän. Genetiska analyser visade att dessa virus blandat sig med influensavarianter som har stor potential för zoonotisk och pandemisk spridning. Inga av de starkt sjukdomsframkallande varianterna påträffades dock.

Campylobacter-analys med PCR gav ett säkrare resultat än bakterieodling under de förutsättningar som gavs vid provtagning i fält. Med PCR kunde vi påvisa campylobacter-bakterier hos 12% av de provtagna personerna. En markant skillnad fanns hos barn under sexton år där 19% var infekterade, jämfört med 8% av de vuxna. I djurproverna återfanns campylobacter med PCR hos 56% av kycklingarna och 72% av grisarna medan det var mindre vanligt hos ankor (24%), kor (5%) och duvor (33%) och inte alls kunde påvisas hos vattenbufflar. Ytterligare analyser visade att riskfaktorer, som att slakta djur, låta djuren gå fritt vid sov- och matplats, äta otillräckligt tillagat kött samt kyckling och fläsk flera dagar i månaden, var associerade till campylobacter-positiva personer i hushållet.

I avhandlingen presenteras nya resultat som belyser behovet av en ökad riskmedvetenhet om zoonoser inom småskaliga jordbruk. Genom riktad provtagning och övervakning av influensavirus och campylobacter kan kunskapen om hur zoonoser överförs förmedlas till regional och nationell nivå. Det kan bidra till förbättrade statliga kontrollåtgärder och biståndsinsatser, vilket i sin tur kan förbättra levnadsvillkoren på Kambodja landsbygd.

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