INFLUENZA A VIRUSES OF SWINE IN WESTERN CANADIAN PIGS AND PEOPLE

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By

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ABSTRACT

Influenza A viruses (IAV) are well-known for their zoonotic potential, and the health and economic threats they pose to humans and pigs. The complexity of influenza virus ecology involving genetically variant viral strains and several natural hosts means that the virus continuously challenges the host-species barrier. Surveillance of IAV is essential as it provides helpful information that can lead to a better understanding of the behavior of the virus at the animal-human interface, the risk factors and the key genetic changes that allow the virus to cross the species barrier. The research aimed to compare the suitability of samples collected for the detection of IAV in swine and to identify the epidemiological and viral factors that might play a fundamental role in the human-swine interface of transmission. The suitability of three types of samples for the detection of IAV in pigs, nasal swabs (NS), oral fluids (OF) and oral swabs (OS), was compared. IAV Matrix gene PCR results showed NS were the most effective method of IAV detection in swine. Compared to NS, OS had a relative sensitivity of 43.6% to 43.8% and relative specificity of 99.3% to 100%. The relative sensitivity and specificity of OF was 57.1% and 95.5%, respectively. Furthermore, the degree of agreement between NS and the other two samples was moderate (k = 0.531-0.583, $p < 0.001$). Human-swine transmission was evaluated through a pilot project consisting of active surveillance in both swine workers and pigs from 11 farms in Western Canada. Nasal swabs, OS, and surveys assessing flu-like symptoms were collected from 26 swine workers and results were compared with Matrix real-time reverse transcriptase PCR (RT-qPCR) results from swine nasal swabs. There was no statistically significant correlation between the clinical symptoms in humans and the RT-qPCR results from swine samples. However, the IAV Matrix gene PCR results from the NS and OS of the swine workers had a very weak correlation with the results found in swine (r = 0.182-0.200, $p = 0.024$-0.040). Transmission among species was not confirmed, but samples with suspect results from human samples coincided with positive swine pool results and the presence of an Alpha H1N2 virus in 4 farms, which is suggestive of a common link between humans and pigs for IAV.

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DEDICATION

To my mother and father, my sister and my brother, and Cami,

For all the sacrifices that you have made on my behalf, as well as, for all your love and support.

To my husband, Derek

For your constant support and love

ORIGINAL CONTRIBUTION

This thesis presents the findings of an independent research project. I contributed extensively to various aspects of this research project, from receiving the samples to preparing the final presentation. Funding was used for the purchase of laboratory utensils and materials associated with the research. My responsibilities included performing some of the laboratory work associated with a research project while collecting and analyzing the data. Prairie Diagnostic Services, Inc. (PDS) provided all laboratory facilities. University of Minnesota Veterinary Diagnostic Laboratory performed the virus isolation and sequencing of influenza A virus.

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LIST OF ABBREVIATIONS

AIV	Avian influenza viruses
APP	*Actinobacillus pleuropneumoniae*
ASFV	African Swine Fever Virus
CDC	Centers for Disease Control and Prevention
CEIRS	Centres of Excellence for Influenza Research and Surveillance
CFIA	Canadian Food Inspection Agency
CMt	Candidatus *Mycoplasma turicensis*
CPE	Cytopathic Effect
CSFV	Classical Swine Fever Virus
Ct	Cycle Threshold
ELISA	Enzyme-linked Immunosorbent Assay
FeLV	Feline Leukemia Virus
FIV	Feline Immunodeficiency Virus
FMDV	Foot-and-Mouth Disease Virus
GISN	Global Influenza Surveillance Network
Gp-340	Glycoprotein 340
HA	Hemagglutinin
HA1	Hemagglutinin Subunit 1
HA2	Hemagglutinin Subunit 2
HI	Hemagglutinin Inhibition
IAV	Influenza A Virus
IAV-S	Influenza A Virus in Swine
IFA	Immunofluorescence
IFN	Interferon
IHC	Immunohistochemistry
ILI	Influenza-like Illness
IgG	Immunoglobulin G
M1	Matrix Protein 1
M2	Matrix Protein 2

MDCK	Madin-Darby Canine Kidney
MUCIN5B	Mucin 5B
NA	Neuraminidase
NEP	Nuclear Export Protein
NICs	National Influenza Centres
NLSs	Nuclear Localization Signals
NP	Nucleoprotein
NS1	Non-structural Protein 1
NS2	Non-structural Protein 2
OIE	World Organisation of Animal Health
OF	Oral Fluids
OPS	Oropharyngeal Swabs
PA	Polymerase Acid Protein
PB1	Polymerase Basic Protein 1
PB2	Polymerase Basic Protein 2
PCV-2	Porcine Coronavirus type 2
PCR	Polymerase Chain Reaction
pdmH1N1	2009 H1N1 pandemic virus
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
RT-qPCR	Real-time Reverse Transcriptase Polymerase Chain Reaction
RBCs	Red Blood Cells
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein Complex
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SN	Serum Neutralization
TB	Tuberculosis
TEGV	Transmissible Gastroenteritis Virus
TRIG	Triple Reassortant Internal Genes
UMVDL	University of Minnesota, Veterinary Diagnostic Laboratory
U.S.	United States
USDA	United States Department of Agriculture

VN	Virus Neutralization
vRNA	Viral Ribonucleic Acid
VSV	Vesicular Stomatitis Virus
VTM	Viral Transport Media
WHO	World Health Organization
WHOCCs	World Health Organization Collaborating Centres
µl	Microliter
µm	Micrometer

1 INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Influenza is a viral disease causing significant health and economic impact on both humans and animals worldwide. Influenza was first recognized clinically in pigs during 1918 in the Midwestern United States, coinciding with the Spanish flu human pandemic (Vincent 2008), and it was first isolated from pigs in 1930 (Shope 1931).

Influenza viruses are classified into A, B, C and D viruses. Of these, influenza A viruses (IAV) are the most widely researched and of zoonotic concern (Koen 1919; Kluska 1961; Zimmer 2009; Vincent 2008). However, most IAVs have a limited host range. Within the IAV genus, there are 18 "H" or Hemagglutinin subtypes and 11 "N" or Neuraminidase subtypes. Two of these matched subtype pairs (H17N10 and H18N11) have only been found in bats (Tong 2012; Tong 2013). The remaining H1-16 and N1-9 can be paired up to create different subtypes, most of which can be found in the natural reservoirs of the influenza virus. The natural reservoirs are species in which all the species-specific strains can be genetically traced back to when the whole viral genome is sequenced and analyzed. Aquatic and shorebirds are considered the quintessential natural reservoir for IAVs (Kapoor and Dhama 2014; Vincent 2009b). For both humans and pigs, endemic strains are of the H1N1, H1N2 and H3N2 subtypes.

The complex properties of influenza viruses continuously challenge the host-species barrier. The frequency of antigenic shift and drift, along with the emergence of novel virulent strains in both animals and humans means that cross-species adaptation and zoonosis is a constant possibility (Lorusso 2011a; Medina and García-Sastre 2011). Occupational exposure of humans to pigs has been shown to increase the risk for influenza A virus in swine (IAV-S) infection. Swine workers, veterinarians and their families are the populations with higher odds of exposure (Gray 2007b; Olsen 2002b). Thus, the apparent frequency with which swine farm workers are exposed to influenza viruses makes the emergence of novel strains in pigs a risk (Olsen 2002b).

The directionality and relative threat of transmission or zoonosis of IAV-S has frequently been assumed to be from swine into humans. The anthroponotic disease theory suggests that introductions occur in the opposite direction; from humans into swine, and could play a role in influenza virus evolution (Nelson 2012b). In this context, the creation of novel influenza viruses

through the reassortment of viruses from humans and pigs are of concern to stakeholders, including public health officers.

Understanding the dynamics of the two-way transmission of IAV-S, the risks of zoonosis, the high rate of evolution of IAV, surveillance in both species, and particularly at the human-pig interface will be critical to understanding both the risk factors and the key genetic changes in the virus associated with interspecies adaptations.

1.2 Influenza A virus

IAV is the predominant type of influenza virus type detected in all species, and many (particularly avian- and swine-origin viruses) have been identified in the surveillance system for their potential cross over to humans. IAV transmission between humans and pigs has been suspected since 1918 when a respiratory disease outbreak was observed in the pigs and the humans in direct contact with them (Paccha 2016a). Since then, multiple single transmission and rare secondary transmission events from pigs to humans have been reported in North America, Europe and Asia (Myers 2007).

1.2.1 Virology

Influenza A viruses represent one of the seven genera of the family *Orthomyxoviridae,* which includes *Influenzavirus A, Influenzavirus B, Influenzavirus C, Influenzavirus D, Thogovirus, Isavirus,* and *Quaranfilvirus* (Kapoor and Dhama 2014; McCauley J.W. 2011; Su 2017). Type B viruses are among the endemic seasonal viruses circulating in people, but rarely there have been documented infections with these types outside of humans (Van Reeth 2007). Influenza C viruses are extremely rare, having only a few strains being detected in humans and pigs and primarily by serology (Guo 1986). Influenza D viruses were more recently established as a separate type and have been detected in cattle and pigs (Hause 2014; Sheng 2014; Su 2017).

The viruses of the *Orthomyxoviridae* family have a segmented negative-stranded RNA genome (Bouvier and Palese 2008; Szewczyk 2014). The eight gene segments of IAV encode for up to 12 protein products. Segment 1 encodes for the polymerase basic protein 2 (PB2). Segment 2 encodes for the polymerase basic protein 1 (PB1), the PB1-F2, and the PB1-N40 proteins. Segment 3 encodes for the polymerase acidic (PA) protein. Segment 4 encodes for the hemagglutinin (HA) protein. Segment 5 encodes for the nucleoprotein (NP). Segment 6 encodes for the neuraminidase (NA) protein. Segment 7 encodes for the two matrix proteins (M1 and

M2). Finally, segment 8 encodes the two nonstructural proteins (NS1 and NS2/NEP) (Bouvier and Palese 2008; Kapoor and Dhama 2014; McCauley J.W. 2011; Vincent 2008; Wise 2009).

The characteristics of the major internal proteins, NP and M1, distinguish IAVs from the other genera. IAV are further classified into subtypes based on the two major surface proteins, HA and NA. Currently, there are 18 different HA and 11 NA described (Kapoor and Dhama 2014; Vincent 2009b), from which only three HA (H1, H2, and H3) and two NA (N1 and N2) have caused human epizootics(Bouvier and Palese 2008).

1.2.2 Pathogenesis

Both human and swine origin IAVs infect epithelial cells of the respiratory tract. Natural killer cells, B cells as well as antigen presenting cells are also suggested to be susceptible to IAV (Manicassamy 2010). The stages of virus replication involve virus attachment, cell entry and virus uncoating, synthesis of messenger RNA (mRNA), post-transcriptional processing of viral mRNA, translation and post-translational processing of viral proteins, and virus assembly and release from the infected cells (Medina and García-Sastre 2011; Szewczyk 2014).

Viral attachment starts with the recognition of the N-acetylneuraminic (sialic) acid from the host cell surface by the virus. There are two types of sialic acid receptors in the host cells. The first linkage occurs when the carbon-2 of the terminal sialic acid binds to the carbon-3 of galactose forming α-2,3 linkages. The second linkage, α-2,6, happens when the carbon-2 of the terminal sialic acid attaches to the carbon-6 of galactose.

In most avian species, IAVs predominantly attach to the gut epithelium through α-2,3 linkages. IAVs predominantly attach through α-2,6 linkages in the respiratory tracts of humans and pigs, and both α-2,6 and α-2,3 linkages are present deep within the respiratory tract of humans and pigs (Ito 1998; Shinya 2006) and allow for alveolar infections with avian influenza viruses (AIV) (Bouvier and Palese 2008). Consequently, both species have had sporadic reports of AIV infections over the last two decades with high-pathogenicity AIV infections being most serious in humans (Abente 2017; Kerkhove 2013).

The different sialic acid conformations may explain the preference of different viral subtypes for a particular species. However, this preference is not an absolute specificity, which can be bypassed by high viral load or by hemagglutinin gene mutations (Szewczyk 2014).

Influenza A virus has been well recognized for its potential to undergo genetic changes that play an essential role in virus infectivity and immunity. There are three evolutionary

mechanisms for IAV mutation; changes due to RNA polymerase errors, reassortment, and recombination (Szewczyk 2014).

The first mechanism, RNA polymerase errors, is due to the high mutation rate of RNA polymerases compared to DNA viruses. The mutation rate of RNA viruses can be up to 10^5 times higher than for a viral DNA genome. Mutations in the viral genome may have neutral, positive or negative effects on the virus viability. Positive mutation or positive selection in genes that create gradual changes in the antigenicity of the HA or the NA proteins has been so-called 'antigenic drift.' The occurrence of antigenic drift may lead to the establishment of new viral variants. Even though the genes of most importance that can undergo antigenic drift are the ones related to HA and NA proteins, other genes for other proteins can be affected (Szewczyk 2014).

Reassortment, the second mechanism of genetic change of IAV, is a more abrupt change. In this process, a rearrangement of viral segments from two genomic variants infecting the same host cell happens, which creates new strains with drastically different properties from the progeny. When the segments involved are the ones codifying for the HA and NA proteins, the reassortment process is called 'antigenic shift.'

Finally, the third mechanism, RNA recombination is the least common. Recombination can be homologous and non-homologous (He 2008). The more common of the two is the non-homologous recombination, which occurs between genes of different segments. For example, during co-infection with a human IAV and a swine IAV, the resulting recombinant PA gene for the swine IAV may retain nucleotides in positions 1-548 but match the human IAV for nucleotide positions 549-2147 (He 2008). The result of recombination events may also convert low pathogenic strains into highly pathogenic (Szewczyk 2014). The effect of IAV genetic changes, no matter its mechanism, may affect the host immunity against the virus and create the potential for epidemics or pandemics.

1.2.3 Clinical presentation

1.2.3.1 Clinical presentation in swine

The clinical presentation of IAV-S in pigs has been described in two clinical forms. The first, and most common are epizootics of respiratory disease, where the onset of the illness is acute. Clinical signs are characterized by fever, lethargy, coughing, dyspnea, sneezing, and occasionally nasal and/or ocular discharge. The disease also presents with reduced food intake which causes tremendous economic hardship for farmers who are trying to reach market weight

in their animals on timelines (Olsen 2002a; Van Reeth 2012). IAV-S has an incubation period between 1 and 3 days with rapid recovery 4 to 10 days after onset. These acute epizootics can disseminate through a barn in 2 to 3 weeks with high morbidity (approaching 100%) and low mortality (<1%) rates (Van Reeth 2007; Vincent 2008). Subclinical infections can also occur and are very common, in which pigs become infected with one or multiple IAV subtypes without showing any signs of disease (Van Reeth 2007).

The second clinical presentation of the disease is as part of a more insidious condition in pigs known as the porcine respiratory disease complex (PRDC). In this form, IAV-S acts in concert with other pathogens that alter the respiratory homeostasis causing chronic respiratory disease and poor animal growth. The other PRDC pathogens include porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2, *Mycoplasma hyopneumoniae, Haemophilus parasuis* and other causative agents of pneumonia (Van Reeth 2012).

Different factors involving the management of farms can increase the risk of infection of pigs with IAV-S. For example, farrow-to-finish and nursery farms are reported to have higher odds of being positive for IAV compared to finishing farms. This is due to the constant presence of susceptible pigs that comes with the regular flow of weaned pigs coming from different sows (Allerson 2014; Corzo 2014). These recently weaned piglets are recognized to maintain, diversify and transmit the virus when they are moved to the growing sites (Chamba Pardo 2017). Furthermore, continuous systems compared to all-in/all-out systems are also known to have a higher chance of maintaining contagious levels of influenza because of the constant addition of susceptible animals (Janke 2013). Other factors contributing to IAV-S infections include high population density, increased replacement rates, lower down period between batches, high prevalence of IAV-S in young animals, movement of pigs, impaired immunity, and poor biosecurity protocols (Allerson 2014; Corzo 2014; Fablet 2013; Rose 2013; Takemae 2011).

1.2.3.2 Clinical presentation in humans

Influenza A virus infections in humans, with human-origin IAV, have been described as having a mild presentation in most healthy people. Infections can range in severity from asymptomatic infections to severe illness. It has been most commonly characterized by upper respiratory disease, with occasional cases presenting gastrointestinal signs (Dacso 1984). Symptoms can go from very non-specific to typical human influenza symptoms, which include; fever, cough, headache, sore throat, and myalgia (Jhung 2013; Shinde 2009; Zambon 2001).

Recently, a series of infections caused by triple reassortant H3N2 viruses in North America have described cases with eye irritation and lower respiratory symptoms (Jhung 2013; Shinde 2009).

Cases of severe pneumonia, failure of different organs and fatal illness are most commonly seen in pregnant women, children under two years of age and people with chronic lung diseases, including asthma. During the 2009 H1N1 pandemic, obesity and diabetes were also identified as risk factors for severe infection (Medina and García-Sastre 2011).

Based on several reports from the Centers for Disease Control and Prevention (CDC) and research at agricultural exhibitions, the presentations of IAV-S infections in humans are similar to human-origin IAV infections. The main difference has been that IAV-S infections usually can be traced to recent exposure to pigs (Bowman 2014; Wells 1991). Although IAV-S has been reported in human infections, the incubation period and time to onset of clinical signs has not been determined. In general, the incubation period appears to be longer than the usual for human seasonal influenza, with a median incubation period of 3.4 days, and a range from 3 to 9 days after the most recent exposure to pigs (Shinde 2009).

1.2.4 Epidemiology of IAV-S

Transmission of IAV-S in pigs occurs through droplets and aerosols and by direct contact with infected animals or through fomites (Van Reeth 2012). In addition, the transport of millions of hogs in countries like the United States (U.S.) due to the integrated market, provides another possible route of introduction of the virus into farms (Vincent 2009a).

Temperature and humidity conditions might influence the transmission of IAV-S. Conditions such as dry and cold temperatures have been described to contribute to more efficient transmission and spread of the virus. This coincides with the regular occurrence of influenza during fall, winter and early spring with two peaks in November-December and March-April. However, during the 2009 H1N1 pandemic the virus behaved differently, in that it spread during summer and early fall. This indicates that the virus can efficiently spread in a naïve population regardless of seasonality (Medina and García-Sastre 2011).

One of the main characteristics of IAV is its capacity to evolve and create new viruses, posing new threats to pigs and humans. Until the mid-1990s IAV infection in North American pigs was caused almost exclusively by classical H1N1 swine viruses. However, in 1998, a severe outbreak of influenza was observed in pigs on farms from North Carolina, Minnesota, Iowa, and Texas. The causative agents were identified to be influenza viruses from the subtype H3N2. Two

genotypes were described. The first one isolated from the outbreaks in North Carolina contained gene segments like human influenza (HA, NA, PB1) and the classical swine (NS, NP, M, PB2, PA) viruses. While the subsequent viruses isolated from Minnesota, Iowa and Texas contained genes from human (HA, NA, PB1), swine (NS, NP, M), and avian (PB2, PA) lineages (Webby 2000; Zhou 1999). This combination of genes from three different species (human, swine and avian) was termed the triple reassortant internal gene (TRIG) constellation. Since then many reassortant events involving viruses with the TRIG cassette have occurred (Karasin 2006; Nelson 2011; Olsen 2002a).

At present, three main subtypes of IAV are circulating in swine populations around the world; these include H1N1, H3N2, and H1N2. Since 1990, evidence has shown introductions of human seasonal H1 and H3 viruses into the swine population.

Three main H1 lineages have been described to circulate in pigs; 1A, 1B, and 1C. Viruses from the H1-1A lineage are closely related to the classical H1 swine virus first detected in 1930, which evolved from the 1918 Spanish flu virus. Currently, circulating strains contain the TRIG cassette with and without genes from pandemic 2009 H1N1 (pdmH1N1). The 1B lineage or human seasonal-like viruses have resulted from multiple introductions of human seasonal H1 viruses. Finally, the 1C group or Eurasian avian-like viruses arise from introductions of IAV of wild bird-origin into swine, which was first documented in 1980. Reassortment of 1A and 1C viruses resulted in the creation of pdmH1N1 (Smith 2009).

For the North American classification of H1, the phylogenetic and antigenic analysis was used to distinguish 1A.1 (α-H1), 1A.2 (β-H1), 1A.3.3.3 (γ-H1) and 1B (δ-H1) clusters. Additional phylogenetic and antigenic subclustering resulted in classes 1A.3.2 (γ-2), 1A.3.3.2 (pdmH1N1), 1A.3.3.3 (non-pandemic γ-1), 1B.2.2.1 (δ-1a), 1B.2.2.2 (δ-1b), and 1B.2.1 (δ-2) (Anderson 2015, Rajao 2018). While all of these groups co-circulate at various levels in North American pigs, one or more clusters/subclusters of viruses dominate during different time points throughout the year depending on the geographic region and season (Lorusso 2011a; Lorusso 2011b). There is also limited cross-reactivity between the clusters and subclusters, due to the antigenic distances (Lorusso 2011a; Lorusso 2011b). Clusters 1A.1, 1A.2 and 1A.3 evolved from the classical H1N1 lineage and had acquired the TRIG cassette. Subclusters 1B.2.1 and 1B.2.2 have the TRIG cassette and also acquired HA and NA genes from human seasonal viruses

around 2003 and 2005 (Nelson 2011). The 1B.2.2 viruses are often H1N2 subtype while 1B.2.1 are often H1N1 viruses (**Table 1.1**).

On the other hand, viruses of the H3 subtype have been divided into viruses from the 3A and the 3B lineages according to their spatial appearance in the global classification system. Lineage 3A corresponds to viruses from Europe while the 3B viruses correspond to the ones arising from North America (Lewis 2016; Webby 2000). The North American classification system for H3 viruses divides these into clusters I, II, III and IV. Viruses from cluster III were most common in North America until they were completely replaced by cluster IV around 2006 (Olsen 2006; Vincent 2008). Cluster IV viruses have continuously evolved and created additional antigenic subclusters IVA to IVF (Lewis 2014).

HA and NA pairing preferences exist. That is, certain HA segments have a clear preference for N1 while others have a preference for N2. H1 1B.2 and H3 viruses usually pair with N2 segments, while H1 1A.3 segments most frequently pair with N1 segments (Nelson 2012a). Viruses of the H1N2 subtype are derived from a recent reassortant event of an H3N2 virus and an H1 HA closely related to the classical swine H1N1 virus (Karasin 2006).

1.2.5 Interspecies transmission

One of the main properties of IAV-S and what makes it a substantial risk for public health is its transmissibility between species. Success for cross-species dissemination depends directly on both host and viral factors (Webster 1992).

The most critical genetic factor of the virus directly involved in cross-species transmission is the binding of HA protein to the sialic acid receptors in the host cell. As mentioned previously in section 1.2.2, pigs and humans have receptors for both mammalian and avian influenza viruses. These are found in the upper and lower respiratory tracts, respectively (Shinya 2006), which makes transmission of IAV possible across species. Also, viruses found to contain D225G mutations are shown to have increased α-2,3 sialic acid binding, which confers dual receptor specificity (Medina and García-Sastre 2011).

Host factors of importance for transmission of IAV from humans to pigs and vice-versa include; the route of virus excretion by the host, contact between infected and non-infected organisms, and immune status of the recipient host (Van Reeth 2007). Contact with pigs has been described as an important factor for IAV-S transmission to humans. Multiple studies have shown the increased risk of influenza-like illness (ILI) in humans with previous contact with

swine. Myers et al. (2007) explored IAV-S cases in humans showing that the majority of patients (61%) reported recent exposure to pigs. Thus, people with increased contact to pigs, such as farmers, meat processing workers, veterinarians, and county fair visitors have an increased risk of developing IAV infections (Nelson 2012a; Vincent 2008; Vincent 2009b). Studies show IAV-S antibodies present in up to 23% of humans with occupational exposure to swine; this risk group also demonstrated to have significantly higher titers compared to individuals with no swine exposition (odds ratio=3.05; 95% CI [1.65, 5.64]) (Paccha 2016b; Van Reeth 2007). Consequently, swine workers not only are at risk of occupational infection but might act as a source of infection for their community and other pigs, as well as contributing to the emergence of novel IAV strains (Gray 2007b; Krueger and Gray 2013).

It is important to emphasize that once a virus is established in a population, IAV might pose a substantial risk for the other species and it may not only spread but allow the generation of novel viral progeny. In the case of humans, the presence of the virus in the population is not enough to cause a pandemic event, the adaptation to the new host and capacity of transmission from person to person is a requirement (Van Reeth 2007). Human to human transmission of IAV-S has rarely been reported.

The human-animal interface behavior of influenza is recognized to be a two-way pathway, with not only introduction of swine viruses into people but also characterized by the introduction of viruses of human-origin into the swine population (Cappuccio 2011; Nelson 2012b; Nelson 2014; Nelson 2015b). Thus, surveillance of IAV should be done in both human and swine populations.

1.2.6 Diagnosis and surveillance

Surveillance is a crucial component in the epidemiology of infectious diseases like influenza. Well-developed surveillance programs permit the understanding of epidemiological patterns, risk factors, burden of disease, emergence of novel viruses, and composition of vaccines. Surveillance provides data to help understand not only the determinants of infections but also the scientific evidence necessary for the development of disease control and prevention plans for both humans and animals (Corzo 2013).

In most countries, human influenza surveillance is more structured and better funded than animal influenza surveillance. For instance, in 1952 an international network of influenza laboratories under the direction of the World Health Organization (WHO) created the Global

Influenza Surveillance Network (GISN). This network included National Influenza Centres (NICs), WHO Collaborating Centres (WHOCCs), and key reference laboratories. The primary objective of this group was to provide detailed virology information about influenza viruses to develop vaccines with the most accurate composition. Currently, the GISN also serves as a primary alert mechanism for novel influenza viruses that can pose a risk to communities and cause a pandemic (WHO 2011).

Even though viral detection and disease data collection and reporting for humans is well established, swine workers who are a population at high risk for IAV-S infections, have not been included in the system. When considering the threat of novel virus emergence, swine workers should be included in surveillance programs as a potential source of infection to the community and other species (Gray 2007b; Myers 2007).

Animal surveillance systems are limited in scope and funding and do not always include IAV-S. As of 2017, IAV has not been included in the list of notifiable viruses (OIE 2017). Limited laboratory and monetary resources, the perceived low impact of IAV on animal health and productivity, and the fear of negative consequences for international trade have created a challenge to IAV-S surveillance (Detmer 2013a). Additional challenges for animal surveillance programs include the frequent presence of respiratory disease in pigs, the lack of specificity of IAV-S clinical signs and macroscopic lesions, the highly mutagenic nature of the virus, the zoonotic potential, and the increased movement of pigs and humans across the globe (Detmer 2013a).

Development of a global animal-human surveillance system for IAV, with a multidisciplinary approach, involving all the stakeholders affected, could provide several advantages. Surveillance of IAV-S will provide the necessary data for the development of required preparedness plans and will improve the strategies for public health protection, including for detection and evolution of influenza, for follow up of novel viruses, and for the development of better diagnostic assays and vaccines.

Two types of surveillance can be conducted; active and passive. Active surveillance is prospective and samples are collected at regular intervals whether or not there are clinical signs of disease. This kind of surveillance provides more representative information about the population and the epidemiology of the virus in swine farms. An example of a group that conducts active surveillance is the Centers of Excellence for Influenza Research and Surveillance

(CEIRS) in the U.S. They conducted an active surveillance program in areas with high swine density (Detmer 2013a; NIAID 2010).

Passive surveillance uses samples collected from clinically ill animals and often produces a higher rate of diagnoses than active surveillance due to the selection process. The United States Department of Agriculture (USDA) conducts large-scale passive surveillance of IAV-S by using samples that are IAV positive at state diagnostic laboratories. The USDA program started in 2009, allowed for anonymous and tracked samples to be isolated and sequenced at a subsidized fee to increase the number of HA, NA, and M gene sequences publically available in GenBank. They also archive and perform whole-genome sequencing on viruses of interest provided by the state diagnostic labs to the National Veterinary Services Laboratories in Ames, Iowa (Anderson 2015, Rajao 2018).

One of the most important tasks when implementing surveillance programs is the selection of diagnostic tools; including diagnostic assays and sampling methods. For detection of IAV-S, multiple diagnostic tests are recognized ranging from histopathologic examination to molecular techniques. The selection of the best diagnostic tool for identification of the virus is challenged by the presence of various strains co-circulating among swine (Webby 2004).

As mentioned previously, because of the nature of IAV-S infection, clinical signs and histopathologic examination are nonspecific. While they can be useful for understanding the pathogenesis of the virus and giving a presumptive diagnosis of influenza, they are not sufficient for a definitive diagnosis (Detmer 2013a). Macroscopic lesions include; multifocal to coalescent consolidation and a purple-red tint, predominantly in the cranio-ventral portions of the lungs. Microscopic changes are characterized by necrosis of the bronchial epithelium with sloughing of necrotic cells into the lumen, accompanied by cellular debris, proteinaceous fluid, and few leukocytes. There is also peribronchial lymphocytic infiltration with various degrees of interstitial pneumonia (Janke 2013; Vincent 2008).

Diagnostic approaches for influenza and other infectious diseases involve the use of either direct or indirect methods of pathogen detection. Direct methods are diagnostic tests capable of detecting the viral antigen, the viral nucleic acids, or the whole virus; for IAV these include virus isolation and RT-qPCR. On the other hand, indirect methods are mostly used in cases where the virus is suspected but is no longer detectable, these types of diagnostic tests detect specific immunoglobulins against the pathogen. Indirect methods described for IAV

include; Hemagglutinin inhibition (HI), Serum neutralization (SN), virus neutralization (VN), and Enzyme-linked immunosorbent assay (ELISA) (Detmer 2013a; WHO 2011).

Direct detection of IAV antigen has been done using two diagnostic assays; Immunohistochemistry (IHC) and Immunofluorescence (IFA). Both tests can be done in frozen or formalin-fixed tissues, and different antibodies can be used. The use of antibodies against the nucleoprotein (NP), permits the recognition of all IAV as the NP is well-conserved among all viruses. Antibodies against HA protein have also been used to identify specific subtypes (Detmer 2013a).

RNA extraction and purification methods are useful for the detection of viral nucleic acids. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) is one of the most used tests as it is an accurate, rapid and sensitive test. It can be used for a variety of sample types. This test can detect a broad range of IAV subtypes, as the primers for the RT-qPCR target the most conserved genes; M and NP. A substantial disadvantage is the incapacity to differentiate between viable and non-viable virus (Detmer 2013a).

The isolation of IAV in cell cultures has been the classic gold standard for diagnosis and acquisition of viable virus. It is usually used in combination with RT-PCR for the detection of virus. The most common cell culture used, because of its high sensitivity to IAV, is the Madin-Darby canine kidney (MDCK) cell (Szewczyk 2014). The advantage of virus isolation is the capacity to amplify the amount of virus in the original specimen; this is helpful for the further antigenic and genetic characterization of the virus. The length of time for this diagnostic process and the technical expertise needed for influenza is a limitation for use in clinical settings (WHO 2011).

Indirect methods are useful when viruses have a short shedding period, like IAV. In this case, detection of specific immunoglobulins may be pertinent. Immunoglobulins, specifically IgG, are detectable in swine between 7 to 10 days with a peak by 2 to 3 weeks post-infection. Antibodies can remain high for several weeks and start to decline 8 to 10 weeks post-infection (Heinen 2000; Van Reeth 2006). It is important to recognize that antibodies can appear after vaccination as well. It is recommended that for the diagnosis of an acute infection of IAV paired samples are taken (Van Reeth 2012).

Hemagglutination Inhibition (HI) is an indirect test that evaluates the presence of IAV by the occurrence of red blood cell agglutination. After exposure or vaccination, anti-hemagglutinin

antibodies are present and provoke the inhibition of red blood cells (RBCs) agglutination by the virus. The HI titer of the sample serum is then the reciprocal of the highest serum dilution that can inhibit HA. HI titers greater or equal to 1:40 are considered protective (Hancock 2009). The low cost, ease, and standardized procedure are advantages of this test (Detmer 2013a).

Serum neutralization (SN) test detects IAV specific neutralizing antibodies present in serum. This assay identifies the highest dilution in which serum neutralizes virus infection and inhibits production of cytopathic effect (CPE). Again, as in the HI, the reciprocal of the highest neutralizing dilution is denominated the SN titer in the specific serum evaluated. This test is advantageous as it demonstrated the biologic neutralization activity of the antibodies in serum. However, the test requires specialized equipment and technical expertise for the viral cultures and results usually take longer than 72 hours (Detmer 2013a).

Another indirect type of test for IAV is the Enzyme-linked immunosorbent assay (ELISA). In this diagnostic method, an anti-influenza monoclonal antibody linked to an enzyme binds to an antigen, producing a color reaction. The color reaction is then read in a spectrophotometer and interpreted using the amount of optical density, which is inversely proportional to the load of antibodies present in the sample. Different commercial ELISA kits are available, some are subtype-specific for H1N1 and H3N2, and others are multiscreen assays that use an NP epitope (Detmer 2013a).

As new strains of IAV are continually emerging, identification of the virus is not the only required procedure, especially in surveillance programs. Virus sequencing and subtyping are essential tools that permit an antigenic as well as a phylogenetic approach necessary for the better understanding of the evolution and geographical relation of IAV. Traditionally, HI assays, as well as NA inhibition tests, were used for detection and subtyping. Currently, new tools such as multiplex and nested RT-PCR provide not only identification but also the possibility for viral sequencing (Detmer 2013a).

The rapid evolution of IAV is an immense challenge. Cross-reactivity between viruses, the constant necessity to produce and validate new primers for RT-PCR are only some of the limitations for diagnosis and surveillance of influenza (Detmer 2013a).

1.2.7 Sample selection

As mentioned previously there are different assays for the diagnosis of IAV; all assays require specific samples for successful identification of the virus. Viral detection is most

sensitive during the febrile period of illness, and a 2 to 4-week lag period is required for serologic titer development (Van Reeth 2012).

Antemortem samples for individual testing include nasal swabs, tracheal swabs, and tracheal fluids. The most common antemortem samples for individual pig testing are nasal swabs because of the reliability and accessibility (Schorr 1994; OIE 2015). Tracheal swabs and tracheal fluids can be collected from live pigs, but they are difficult to collect on conscious pigs. For population-based studies, oral fluids have been reported as a good alternative and have been heavily studied in pigs since 2008. Environmental samples such as air specimens are also crucial for surveillance studies (Detmer 2013a; WHO 2011) but require special equipment. From all these antemortem samples, nasal swabs are the gold standard sample for IAV identification and are used to collect samples from live pigs for most surveillance studies (Corzo 2013; Janke 2000; Schorr 1994).

Postmortem samples from animals that die or are euthanized in the acute phase of the influenza disease are very useful for diagnosis. Lung tissue is the most common and reliable sample for both detection and isolation of IAV-S for diagnostic case submissions (Janke 2000; OIE 2015; Swenson 2001). However, nasal turbinates, trachea, and bronchoalveolar lavage fluid can be used. For microscopic examination of IAV-S lesions, tonsil and bronchial lymph nodes are also recommended in addition to lung, nasal turbinates, and trachea. Diagnostic tests such as IHC and IFA can be performed in these tissues after fixation as well (Detmer 2013a; Olsen 2013; WHO 2011).

1.3 Nasal Swabs

Nasal swabs (NS) are considered the gold standard antemortem sample for any number of respiratory and systemic pathogens where nasal shedding is part of the clinical picture. The reliability of nasal swabs for pathogen detection dates back decades before PCR assays were developed when virus and bacterial isolation were the standard methods of detection in humans and animals. Influenza viruses have been one of the most studied respiratory viruses in the last century. Progressing from using embryonated chicken eggs and lung tissue homogenates for the first strain of IAV-S in 1930 (Shope 1931) to the first detections using reverse transcription PCR for animal influenzas (Spackman 2003; Quinlivan 2004), nasal swabs and lung samples have been the primary samples used over the last century.

In humans, NS have been used for the diagnosis of different respiratory infections including; Human Metapneumovirus (Mullins 2004), Respiratory Syncytial Virus (Falsey 2002), Rhinovirus (Ali 2015) and influenza viruses (WHO 2011). Nasal swabs have also been used in healthcare and community settings for the culture of *Staphylococcus aureus* (Warnke 2014).

1.3.1 Nasal swabs in veterinary medicine

In the current practice of veterinary medicine, the use of PCR assays for pathogen detection is an ever-present procedure for a disease investigation. These assays are typically developed using purely isolated pathogens or artificially constructed RNA/DNA, and then validated for sample types where the pathogen is expected to be found based on knowledge of pathogenesis (e.g., serum, whole blood, swabs, or tissue).

The use of nasal swabs for pathogen detection using PCR assays is pervasive in veterinary medicine because NS can be used to detect a wide range of pathogens that include, but are not limited to Foot-and-Mouth virus (Moniwa 2007), bovine respiratory disease organisms (Moore 2014), and equine herpesvirus type 1 (Hussey, 2016). In pigs, the use of NS has been applied for diagnosis of *Mycoplasma hyopneumoniae* (Calsamiglia 1999), Porcine Circovirus type 2 (Shibata 2003), and Porcine respiratory coronavirus (Van Reeth 1996).

The adoption of NS as specimens for diagnosis has been broadly used for influenza in multiple species including pigs (Corzo 2013; Janke 2000; Schorr 1994), dogs (Hong 2013) and horses (Chambers 2014; Galvin 2014).

1.3.2 Nasal swab sampling and diagnostics

For active surveillance, where the animals are typically not sacrificed, the sample must be relatively easy to collect with minimal restraint and cause minimal discomfort. For sample collection, pigs are restrained with the head positioning upward to have comfortable and optimal access to the nasal cavity. Once the animal is in position, a sterile swab is inserted into the nasal cavity while gently swabbing the surface of the nasal mucosa. It is recommended that this same procedure is repeated for both nostrils with the same swab (USDA 2012). It is also important to note that there are several options for swabs. The main two swab types used for IAV-S include either flocked or smooth tipped rayon or dacron material. These can have either a plastic or metal stem. Side-by-side comparisons of flocked and dacron swabs with plastic stems have demonstrated no significant differences found in PCR detection rates (Gramer 2007). The use of

a viral transport media is also recommended for these samples to prolong the viability of the virus for culture (Culhane and Detmer 2014).

The sample must also be validated for the assay used with reasonable sensitivity and specificity. Some of the earliest diagnostic work with IAV and PCR used nested PCR methods for the NP gene (Oxburgh 1999, Tsuruoka 1997). The original NP gene PCR assay was not as sensitive as was desired and the Matrix gene assay was developed shortly thereafter (Fouchier 2000, Spackman 2003). While initially developed for AIV, Matrix PCR assays are also reliable for other species with a universal target in the Matrix gene (Fouchier 2000).

Nasal swabs were initially validated for IAV-S using the NP PCR, but sensitivity was an issue (Gramer 2007). This issue was overcome with the Matrix PCR assay, and together with NS, these two are currently considered the gold standard for surveillance in live pigs for the detection of IAV-S (Corzo 2013; Janke 2000; Schorr 1994). However, limitations including the cost of individual sample testing and labor needed to acquire this sample can make NS not the most cost-effective way to monitor a population for disease in situations where only PCR detection of the disease is required (Panyasing 2016).

1.4 Oral Fluids

The use of oral fluids (OF) as a diagnostic sample is increasing in both humans and animals (Detmer 2011; Olsen 2013; Prickett 2008b; Ramirez 2012). This type of specimen is practical and cost-effective, which can facilitate monitoring and surveillance of pathogens that have economic significance for swine production and humans (Olsen 2013; Ramirez 2012).

OF is a mixture of saliva with mucosal transudate, bronchial and nasal secretions, and can contain inflammatory components, bacteria, viruses, and epithelial cells (McKie 2002; Romagosa 2012). It is obtained by the introduction of absorptive collectors into the mouth (Olsen 2013; Prickett and Zimmerman 2010), of which cotton materials are the most commonly used collective material (Olsen 2013). It is different from ‘whole saliva’ which is the fluid obtained by expectoration (Atkinson 1993).

The OF sample itself can be impacted by the method of collection, the collection material, and the site of the collection in the oral cavity (Atkinson 1993). A major limitation to diagnostic use of OF is the presence of multiple inhibitors that affect the sample quality and the test result, particularly when using PCR (Hernandez-Garcia 2017). As for virus isolation from

OF, microbial, fecal and food contaminants can be difficult to filter out in swine OF and can also result in cell culture contamination.

The use of OF in animal health, as in human health, has been described as a useful tool for monitoring, surveillance, and detection of diseases (Prickett and Zimmerman 2010). Since 1985 there was a hope that this specimen could serve as a tool for disease prevalence surveys to monitor the health of animals, especially production animals, in an attempt to anticipate infectious pathogens before epidemics could occur (Ramirez 2012). Since then, OF sampling and testing has met this objective and has demonstrated its usefulness for the identification of antibodies, pathogens and antimicrobials in populations (Detmer 2011; Prickett and Zimmerman 2010; Ramirez 2012).

In humans, OF are easily collected and have been used for both viral and antibody detection as well as for drug and hormone level monitoring (Hernandez-Garcia 2017; Prickett 2008b). Viruses identified in OF human specimens include Hepatitis A virus, Hepatitis B virus, Human immunodeficiency virus, and Measles virus, to name a few. Antibodies against Dengue virus and others have also been demonstrated in OF (Prickett 2008b).

1.4.1 Oral fluids in veterinary medicine

The application of OF-based testing has been used in different animal species. In cats, it has been used for the detection of Feline leukemia virus (FeLV) (Lewis 1987), Feline immunodeficiency virus (FIV) (Yamamoto 1988), and '*Candidatus* Mycoplasma turicensis' (CMt) (Willi 2006). In dogs, OF are useful to detect Rabies virus and *Bartonella* spp. (Côrtes 1979; Duncan 2007). In horses, the primary use is for testing the presence of performance altering chemicals in racehorses (Morgan and Gellhorn 1947). In cattle, detection of bacterial and viral pathogens has been done, including *Escherichia coli*, *Salmonella* and Picornavirus (Archetti 1995; Prickett and Zimmerman 2010; Smith 2004).

In pigs, oral fluids have become a popular method for the detection of several pathogens. It is cost-effective, non-invasive and it takes advantage of the pigs' natural instinct to investigate new objects within their pen by chewing and depositing oral fluids on cotton ropes (Prickett 2008b; Romagosa 2012). Pathogens detected using OF in pigs include influenza A virus (IAV), classical swine fever virus (CSFV), transmissible gastroenteritis virus (TEGV), vesicular stomatitis virus (VSV), foot-and-mouth disease virus (FMDV), porcine respiratory and reproductive syndrome virus (PRRSV), porcine circovirus type 2 (PCV-2), and *Actinobacillus*

pleuropneumoniae (APP) (Detmer 2011; Prickett 2008a; Prickett 2008b; Prickett and Zimmerman 2010; Romagosa 2012).

1.4.2 Oral fluids sampling and diagnostics

In pigs, OFs are collected using suspending ropes set-up in the pen. The animals chew on the ropes thereby depositing oral fluids on them (Prickett 2008b). Ropes are placed at shoulder height in a clean area away from feed and water. One rope per 25 pigs is recommended (CFSPH 2015). As pigs are more active during morning hours, it is recommended to do the collection at this time, as doing it in the afternoon may prolong the time needed for sample collection (CFSPH 2015). Younger, untrained pigs may be challenging to collect. For these cases, training ropes with sugar solution could be placed in the pen, and subsequently, pigs will chew more aggressively in the diagnostic sample ropes (CFSPH 2015; Hernandez-Garcia 2017). Ropes remain in the pens for 20 to 30 minutes to provide exposure to as many pigs as possible and to recover a minimum of 5 ml of oral fluids (CFSPH 2015; Detmer 2011). Post-collection the ropes are compressed and subsequently harvested to obtain the oral fluid for further testing (Olsen 2013).

OF offers multiple benefits for population-based monitoring and surveillance of IAV-S in pigs. Using a population-based approach and targeting as many pens as possible, provides a sampling design that can identify IAV symptomatic as well as asymptomatic pigs (Corzo 2013; Hernandez-Garcia 2017).

The limitations to OF sampling are that feed, and fecal material can act as inhibitors of the detection of IAV-S in OF samples, making it less possible to detect the virus in the specimen by using a PCR technique. Other materials can also serve as inhibitory agents. In humans, salivary glycoprotein-340 (gp-340) and mucin 5B (MUC520) have inhibitory and neutralizing effects against IAV strains. Gp-340 present in saliva and bronchoalveolar lavage fluid directly inhibits the binding of the sialic acid receptor with the viral HA. MUC5B is believed to have a similar inhibitory activity of the HA. Even though these proteins have been studied in humans, there is no information about their presence and activity in swine saliva (Detmer 2011; White 2009). Another disadvantage of using OF for IAV-S diagnosis is the limited ability to isolate the virus from this specimen. Isolation of viruses is essential for viral sequencing and vaccine production (Detmer 2011).

1.5 Oral Swabs

During recent years, oral swabs (OS) as a sampling method has been explored as a diagnostic tool for pigs. OS have started to be used in both experimental and epidemiological field studies (Stenfeldt 2013). OS are an attractive alternative for sampling, as they are easy to obtain, are non-invasive, and there is less specific training required (Icenhour 2001; Stenfeldt 2013). Oral swabs, relative to sputum or OF, have a very small volume and more uniform in volume and composition, while being less viscous and heterogeneous, which make it a good sample alternative (Wood 2015).

This sampling technique has been used in humans for passive surveillance of tuberculosis (TB) as well as for diagnosis of TB in individuals that do not actively produce sputum (Wood 2015). OSs in humans also have been studied as an alternative method for human leishmaniasis infections (Ferreira 2013). A study using a guinea pig model looked at OS as an alternative sample for the antemortem diagnosis of Ebola virus, but further research is still required (Spengler 2015).

1.5.1 Oral swabs in veterinary medicine

The utility of OS, with its simpler approach, has become an attractive alternative for animal infectious diseases surveillance, including Foot and Mouth Disease Virus (FMDV), and *Pneumocystis carinii*, among others. Experimental and epidemiological studies have shown the ability of OS to detect the excretion of FMDV in both cattle and pigs (Mohamed 2011; Stenfeldt 2013). Detection of the virus in OS is reliable during the acute phase of infection, which makes this specimen recommendable for initial analysis of the disease when acute illness is suspected (Stenfeldt 2013).

In rats, OS PCR detection of *P. carinii* has been reliable and has become a promising method that could be applied to other respiratory pathogen models in humans. This specimen has been used not only to determine the exposure of rats to the pathogen but also to confirm the widespread prevalence in commercial rat colonies (Icenhour 2001).

The use of OS in the identification of pig pathogens is uncommon and poorly described. Pathogens detected using OS include, Porcine Circovirus type 2 (PCV2), *Streptococcus suis*, African Swine Fever (ASF), and influenza (Amass 1997; Gabriel 2011; Mantilla Garrido 2017; Shibata 2003).

1.5.2 Oral swab sampling and diagnostics

Very few reports describe the appropriate technique or a full protocol for OS collection. However, OS sampling has been conducted with nasal swabs (Mantilla Garrido 2017; Strobel 2016). No information about collection time points for OS samples is available, but as with other sampling methods, samples might be collected during the first three days of infection in animals that are febrile and have clear nasal discharge.

For deep OS the mouth of the animal must be opened with the help of an assistant and a mouth gag. The swab must be inserted, and vigorously passed backward and forward in the back of the oropharynx near the tonsils. The soft palate should be swabbed for at least 5 seconds (Maddie's Fund 2012). OS in the diagnosis of IAV-S can be a potentially valuable and novel tool for detection and isolation on a site basis study, but further research and validation of this sampling method is needed.

1.6 Aims and Objectives

The overarching aim of this research was to examine the clinical and viral activities of IAV-S at the human-swine interface in pigs and swine workers from selected farms in Western Canada with sampling conducted over a prescribed time period. While the first study (Chapter 2) was based on repeated sampling from a single farm, the purpose of this project was to compare sampling techniques in a natural disease outbreak situation. The aims of second study (Chapter 3) were to compare repeated sampling of both humans and pigs over time along with human surveys and to provide relevant data needed for planning much larger surveillance work at the human-pig interface.

The described risk of infection and transmission of IAV-S for both humans and pigs, the limited information on the present strains of IAV-S in pigs across Western Canada, and the lack of a preferred method for sample collection, makes surveillance programs important. Looking at the swine-human interface then constitutes not only an essential part of the monitoring of influenza, but it also helps in the prevention of significant outbreaks. Swine workers may be an important variable in the transmission process (Myers 2007; Nelson 2012a; Vincent 2009a; Vincent 2008) and should be carefully monitored because of their close proximity to infected animals.

1.6.1 Objectives

The zoonotic potential of IAV-S and the need for early detection and prevention makes surveillance in both humans and pigs essential worldwide. Determining not only the characteristics of the viruses that are circulating but finding alternative and strategic methods for its detection should be a fundamental goal in the study of influenza.

The research in this thesis had three objectives:

1) To compare nasal swabs, oral fluids, and oral swabs as samples for Matrix PCR assays and determine which sample type provides better sensitivity and specificity for the detection and surveillance of Influenza A in pigs.
2) To determine what IAV-S strain types are circulating in pigs on the selected farms in Western Canada and monitor for new virus introductions through active surveillance.
3) To examine the presence and clinical presentation of IAV-S in swine workers and the existence of this virus in pigs of the same farms to explore evidence of transmission between these two species.

Table 1.1 Classification of H1 influenza A viruses with locations where they have been reported *(Adapted from Anderson 2016).*

Lineage (1st order)	Clade (2nd order)	Sub-Clade (3rd order)	4th order	Locations
Classical Swine	1A.1 (α-H1)	1A.1.1		Canada, USA, Taiwan
		1A.1.2		Thailand
		1A.1.3		China, Hong Kong
	1A.2 (β-H1)			USA, Canada
	1A.3 (γ-H1)	1A.3.1		Mexico, USA
		1A.3.2 (γ-2-H1)		Mexico, USA
		1A.3.3	1A.3.3.1	China, Hong Kong
			1A.3.3.2 (H1N1pdm09)	Global
			1A.3.3.3 (γ-H1)	South Korea, Mexico, USA
Human Seasonal	1B.1 (H1N2 GB 1994)	1B.1.1		UK
		1B.1.2	1B.1.2.1	Belgium, Germany, Italy, Netherlands
			1B.1.2.2	Italy
			1B.1.2.3	France
	1B.2 (δ-H1)	1B.2.1 (δ2-H1)		USA, Canada
		1B.2.2 (δ1-H1)	1B.2.2.1 (δ1a)	USA, Canada
			1B.2.2.2 (δ1b)	USA, Canada
Eurasian avian	1C	1C.1		UK
		1C.2	1C.2.1	Belgium, Denmark, France, Germany, Italy, Netherlands, Poland, Spain
			1C.2.2	France, Germany, Netherlands, Poland, Spain
			1C.2.2	China, South Korea

2 COMPARISON BETWEEN THREE SAMPLING METHODS FOR THE DIAGNOSIS OF INFLUENZA A VIRUS IN SWINE

In this study, we investigated different sampling methods for diagnosis of influenza A virus in pigs on a farm in western Canada. This was an initial assessment to determine the best specimen for the diagnosis and future surveillance of influenza in pigs.

Full citation: Murcia-Rodriguez DM, Harding JCS, Kirychuk S, Detmer SE (2016). Comparison between three sampling methods for the diagnosis of influenza in swine.

Author Contributions: Detmer SE and Moshynskyy I conducted the sample collection and laboratory testing for this research. Murcia DM, Harding JCS, Kirychuk S and Detmer SE contributed to conception and design of the study, acquisition, analysis, and interpretation of the data, and writing of the manuscript.

2.1 Abstract

Influenza A virus (IAV) is the causative agent of an infectious disease affecting both animal and human populations. Due to its impact on health and economics, monitoring programs are an important tool. Surveillance of IAV provides helpful information that could improve diagnostic sampling techniques and strategies. This study compared the use of nasal swabs (NS), and two less invasive sampling techniques, oral fluids (OF) and oral swabs (OS), to determine the usefulness of these methods in monitoring IAV in swine (IAV-S). The three sample types were collected throughout this study between March and September 2016 from a farm with a recent introduction of a new IAV-S strain. Six sows (parity 1 or 2) with 2 piglets from each sow, plus an additional 12 nursery pigs were randomly sampled on the farm each month of testing. Matrix RT-qPCR was performed on the samples. IAV was detected in 28/178 (15.7%) of NS, 10/58 (17.2%) of OF, and 12/179 (6.7%) of OS. Sequencing results revealed that the initial introduction of 2009 pandemic H1N1 (pdmH1N1) into the farm studied, just prior to the start of the study was followed by the emergence of a type IV H3N2. Viral detection was successful using all three sample types. However, the rate of detection of IAV as well as the sensitivity and specificity of detection was higher in NS, followed by OF. Further research is necessary to evaluate the usefulness of OF and OS in IAV-S detection including the development of improved protocols using these samples for population surveillance.

2.2 Introduction

Influenza A virus (IAV) is a zoonotic viral disease that represents a health and economic threat to both humans and animals. IAV in contemporary commercial swine populations is an endemic disease characterized by an acute respiratory infection with low mortality rates but a high morbidity presentation which accounts for a decrease in production parameters (Vincent 2008).

In humans, global surveillance programs for IAV are well established; however, global swine surveillance is less consistent. Surveillance is a useful tool as it provides data that will help in the understanding of the determinants of infections, providing enough scientific background for the development of disease control and preventions plans, as well as the development of better diagnostic tools (Corzo 2013). The importance of herd monitoring and surveillance of IAV in the swine population is a result of the viral properties that permit genetic and antigenic variability of this virus and the presence of multiple co-circulating strains (Detmer 2011). IAV surveillance in pigs can be improved if simple, efficient, inexpensive, and reliable sample collection methods are available.

Currently, the standard sample and the gold standard, for detection of IAV in swine (IAV-S) are nasal swabs (NS) (Corzo 2013). This specimen type, although a reliable sample for detecting and isolating IAV-S, can be stressful for pigs and is labor-intensive.

Less commonly used than NS, oral fluids (OF) have gained popularity for swine surveillance because they are easy to collect, not stressful for the pigs, and less labor-intensive (Detmer 2011; Olsen 2013; Prickett 2008b; Ramirez 2012), which can facilitate monitoring and surveillance for swine populations (Olsen 2013; Ramirez 2012). OF are less stressful on the pigs as the method takes advantage of the pigs' natural instinct to investigate new objects within their pen while chewing and depositing oral fluids on ropes (Prickett 2008b; Romagosa 2012). Swine pathogens including IAV-S, porcine respiratory and reproductive syndrome virus (PRRSV), porcine circovirus type 2 (PCV-2), and porcine endemic diarrhea virus (PEDV) have been detected using this sampling method (Detmer 2011; Prickett 2008a; Prickett 2008b; Prickett and Zimmerman 2010b; Romagosa 2012). However, OF have the significant disadvantage of having low to null isolation rate of IAV-S in cell culture (Detmer 2011; Romagosa 2012).

Alternative to NS and OF, a recently proposed diagnostic specimen for the diagnosis of IAV-S is oral swabs (OS). OS are an attractive alternative sample as they are easy to collect,

require less restraint, and there is limited training required (Icenhour 2001; Stenfeldt 2013). Additionally, OS have a very small volume compared to OF and samples are more uniform in volume and composition while being less viscous and heterogeneous (Wood 2015).

This study examined three sampling methods by comparing the use of nasal swabs and two less invasive sampling techniques (OF and OS) to determine the usefulness of oral fluids and oral swabs in monitoring Influenza A in swine. The sensitivity and specificity of OF and OS against the gold standard, NS, for detection of Influenza A virus in pigs were examined.

2.3 Materials and methods

2.3.1 Ethics statement

The animal sample collection was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use (permit #20120097).

2.3.2 Farm and animal selection

A 1200 sow farrow-to-finish farm in central Saskatchewan, Canada was selected. There was an outbreak of respiratory disease confirmed to be caused by the 2009 pandemic H1N1 (pdmH1N1) a few weeks before the start of the study. Sows and gilts were vaccinated with a commercial IAV-S vaccine containing influenza A/CA/07/2009 (Zoetis Animal Health, Quebec, Canada). Six sows (parity 1 or 2) with two piglets per sow, six 6-week-old, and six 8-week-old nursery pigs were randomly selected each month for sample collection.

2.3.3 Sampling collection and processing

Since the introduction of pdmH1N1 to a farm can result in an acute epizootic event lasting as short as two months or the virus can become endemic and genetic changes in the virus can be detected within six months, the samples were collected over a seven month period. Monthly samples from March to September 2016 were collected from all animals selected. Specimens were collected by trained farm personnel and included NS, OF, and OS. Nasal swabs were collected using flocked swabs with viral transport media (VTM); regular swabs for sows and mini swabs for piglets (Puritan Diagnostics, Maine, USA; UT-116 and UT-361, respectively). The oral swabs were collected using rayon-tipped, transport culture swabs (Canadian BD, Mississauga, Ontario, Canada; BD 220099). The oral fluids were collected using 30 cm long cotton ropes hang in the pens with 20 pigs, as previously described by Detmer (2011).

Nasal swabs were vortexed for 10 s, and VTM was transferred to a 2 ml cryovial. Oral swabs were cut into a sterile test tube containing 1.5 ml of VTM and vortexed for 10 seconds before transferred to a 2 ml cryovial. Samples were initially tested in pools (3 NS or 3 OS per pool in 500 μL total), and all pools with a Ct <38 were subsequently tested individually. Oral fluids were centrifuged at 9000 x g for 10 min, decanted into 5 ml tubes as described previously (Ramirez 2012); 500 μL of the supernatant was collected for individual sample testing. Samples were stored frozen at -80°C. The RNA was extracted from 500 μL of the sample according to manufacturer instructions using a commercial kit (Qiagen; Maryland, USA; RNeasy Kit; 74106).

2.3.3.2 Real-time RT-PCR assay

A commercial RT-qPCR kit (Life Technologies, Texas, USA, 4415200) was used according to manufacturer instructions to detect the IAV matrix gene in 8 μL of extracted RNA. For all RNA extracts with a Ct value of 38 and under, a commercial real-time RT-PCR kit (Life Technologies, Texas, USA; 4485541) was used according to manufacturer instructions to detect H1, H3, N1 and N2 genes for subtyping. For both assays, Ct values of 38 or less were considered positive and values equal to or greater than 38.01 were considered negative. The PCR cut-off values were established and validated by the kit manufacturer and Iowa State University Veterinary Diagnostic Laboratory (Spackman 2014, Zhang and Harmon 2014).

2.3.3.3 Virus isolation (VI)

Virus isolation was attempted on individual samples from one pool per month with the lowest Ct (if below 32) at the University of Minnesota Veterinary Diagnostic Laboratory (UMVDL) as previously described (Detmer 2013a).

2.3.4 Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 25 Software. Frequencies for the Matrix RT-qPCR results were calculated. Results of the PCR were categorized into positive and negative, which were used to compare the sample methods. Cross-tables were used to calculate the relative sensitivity and specificity of OF and OS, compared to NS. A Cohen's kappa analysis was performed to evaluate the degree of agreement between the three types of samples.

Calculation of the relative sensitivity, relative specificity, and measure of agreement (Kappa) was done at the pig-level for OS, and at the pen-level for both OF and OS. For pen-based analysis, a pen was classified as positive when at least one of the samples was positive.

Missing cases were not included in the analyses. Furthermore, the relative sensitivity and specificity, and the Kappa value were calculated separately for farrowing and nursery, in addition to the overall population analysis.

A heat map using color coding for the PCR results and listing the different subtypes per month, per sample type, and per stage of production was created to visualize the positive (red) and negative (green) Matrix RT-qPCR results. The farm was considered positive for a month when at least one sample for that farm and sample type was positive.

2.4 Results

2.4.1. Sample collection

Between March and September 2016, 415 samples were received. Samples included a total of 178 individual nasal swabs, 179 individual oral swabs, and 58 oral fluid samples (24 individual sows and 34 nursery pens). Throughout the study, 12 oral fluids, 2 nasal swabs, and 1 oral swab were not received. April and July were the months with more samples missing, with 5 and 4 samples not received, respectively. The sampling that was planned for August was completed in September (**Table 2.1**).

2.4.2. Real-time PCR (RT-PCR) assay

For all the 415 samples collected, the frequency of positive samples was higher for the NS (28/178; 15.7%), compared to the results of OF (10/58; 17.2%) and OS (12/179; 6.7%) that were PCR positive (**Table 2.2**). During April, July, and September IAV-S was detected in the three kinds of samples tested. May had positive results in the NS and OS, whereas samples from March were only positive using NS. June, on the other hand, showed no positive samples. During farrowing, detection of IAV-S did not occur in two of the months of the study (April and June), whereas for nursery IAV-S was detected in all the months studied but June (**Figure 2.1**).

2.4.3. Subtyping analysis and Virus Isolation

Subtyping was successful on samples with a Ct less than 38, except in March when. subtyping was unsuccessful. The whole-herd vaccination occurred in February and March (2 doses) with a commercial pandemic H1N1 virus vaccine, and the pandemic H1N1 virus was not detected after April 2016 (**Figure 2.1**).

Virus isolation was attempted on positive NS pool samples (individual samples per lowest positive pool per month) with a Ct value under 32. These requirements were met in

March, April, May, July, and September and virus isolation was successful on at least one sample each month. A total of 8 isolates were obtained from 13 samples.

2.4.4. Relative sensitivity and specificity of oral fluids and oral swabs

A summary of the general relative sensitivity and specificity, as well as these same calculations for farrowing and nursery, separately is shown in **Table 2.3**. Compared to the gold standard nasal swabs, oral swabs at the individual-level had a relative sensitivity of 32.1% and a relative specificity of 98%. At the pen-level, the relative sensitivity and specificity of oral swabs were 35.3% and 98.2%, respectively. Alternatively, the pen-level relative sensitivity of oral fluids was 60%, and the relative specificity was 97.7%.

In farrowing, the relative sensitivity of OS at an individual-level was 31.6%, and relative specificity was 96.6%. OS, as pen-based samples, had a relative sensitivity of 45.5% and relative specificity of 96%. OF from the individual sows showed a relative sensitivity and specificity of 60% and 100%, respectively.

The relative sensitivity and specificity of OS as individual-based samples for nursery pigs were 33.3% and 100%, respectively. For this same sample but analyzed as a pen-level specimen, the relative sensitivity was 16.68%, and the relative specificity was 100%. For OF in nursery pigs, the values of relative sensitivity and specificity were 60% and 96.2%, accordingly.

2.4.5. Measure of agreement (Kappa) between sample types

At an individual sampling level, based on the Kappa statistical test, a poor degree of agreement between the nasal swabs and oral swabs was detected (**Table 2.2**). The inter-rater-reliability analysis at the pen-level showed a fair degree of agreement between the nasal swabs and the oral swabs, while it was moderate between the nasal swabs and oral fluids (**Table 2.2**).

In farrowing, the degree of agreement of individual-based OS to individual-based NS was still poor. The degree of agreement between pen-based OS and OF compared to pen-based NS was fair and moderate, respectively. The inter-rater-reliability analysis for the nursery samples showed a fair degree of agreement between the individual-based OS and the individual-based NS, while the two at the pen-level was found to be poor. OF showed a moderate degree of agreement when compared to the pen-based NS (**Table 2.2**).

2.5 Discussion

Surveillance of swine for IAV is essential to monitor the evolution and introduction of viral strains as well as for the development of improved diagnostic techniques, including

enhanced sampling methods. Historically, monitoring programs of IAV-S in pigs have used nasal swabs as the gold-standard sample (Corzo 2013). However, the cost of individual sample testing and labor needed to acquire this sample may not be the most cost-effective way to monitor a population for disease (Panyasing 2016). In this study, the use of nasal swabs and two other alternative sampling types (OF and OS) for the diagnosis of IAV-S in pigs were examined on a farm following an outbreak with a new strain of IAV-S.

The presence of IAV-S RNA by Matrix gene PCR was confirmed at a higher rate in nasal swabs than in oral fluids or oral swabs. Nasal swabs identified 28 positive samples while oral fluids and oral swabs were only able to identify 78.6% (22/28) of the positive samples. Relative sensitivity and specificity of pen-level OF compared to the gold standard NS was estimated to be 60% and 97.7%, respectively. This differs from inoculation experiments in controlled research facilities where the overall sensitivity of OF was calculated to be above 80% even in scenarios with a low within-pen prevalence (Detmer 2011; Romagosa 2012). For the pen-level OS, the relative sensitivity and specificity was 35.3% and 98.2%, respectively. By contrast, another field-based study estimated sensitivity for detection of IAV-S in OS of 77% (Strobel 2016). Additionally, individual-level analysis of OS had a lower relative sensitivity (32.1%) and lower specificity (98%) compared to the pen-level estimations. Our results show that individual OS detection of IAV-S is less efficient than any other of the methods evaluated in this study, and they disagree with another report that shows individual samples of OS were superior to NS and OF (Mantilla Garrido 2017).

This study further highlights that the effectiveness of the sampling method had similar results in the different stages of production. For both farrowing and nursery stages, the most sensitive and specific type of sample was NS followed by OF. These results are similar to a previous report showing OF as a better sample compared to OS during farrowing (Strobel 2016), but they differ from another study that demonstrated individual-based OS as the most efficient sample for farrowing pigs (Mantilla Garrido 2017). It is important to clarify that due to the difficulties in the collection of OF in young animals, the OF samples from farrowing corresponded to the sows and not the piglets, which might have implications in the calculations of relative sensitivity and specificity for this sample in this stage of production. The discussion of the variability in the effectiveness of OF collection according to the age of the pigs has

previously been discussed by other authors and has proven to be challenging and to require previous training of the piglets to get better samples (CFSPH 2015; Hernandez-Garcia 2017).

The agreement of OS and NS was slight to fair at the individual- and pen-levels, respectively. While the inter-rater-agreement for OF against NS was moderate. Our results are like other reports showing OS to have a fair degree of agreement (Strobel 2016) but they differ from studies showing an excellent agreement of OF with NS (Romagosa 2012). Findings in our study also highlight the better degree of inter-rater reliability in general and during farrowing of OF and OS when the pen is analyzed as a whole instead of as individual samples. But they show how in nursery aged pigs, OS seemed to work better when analyzed as individual samples. The inter-rater reliability between NS and OS was better in the farrowing stage than in the nursery stage. This was likely due to the fact that farrowing samples included OF only from the sow and not from the young piglets. In nursery pigs, a higher degree of agreement between OF and NS was observed, this might be as well related to the fact that OF samples were taken from all the pigs present in the pen.

The study farm presented with a pH1N1 outbreak a few weeks before the beginning of the study. During the study the H1N1 virus was detected, which is consequent with the virus previously reported. The second strain was an emergence of a type IV H3N2. These findings are consistent with studies showing the presence of both H1N1 and H3N2 viruses in western Canada (Nelson 2017).

Virus isolation in OF and OS has shown variable results and is still a controversial field of study. In this study, only two NS samples fulfilled the requirements that were established *a priori* for subsequent virus isolation, that is, a maximum of one sample per month with a Ct value under 32. This criterion differs from another study which used samples with Ct values under 35. This may account for the difference with our results in which studies have demonstrated success rates for both OF and OS with a high yield of positive results of even 50% (Mantilla Garrido 2017; Romagosa 2012), while others that include only OF have unsuccessfully shown positive VI results (Detmer 2011; Panyasing 2016).

Circumstances such as the amount of virus present on the farm, collection technique, sample transit time, sample processing, and frequency of collection, may have influenced the results of this study. For instance, low amounts of virus result in low RNA recovery and detection of IAV-S in swine (Vosloo 2015). Collection technique is also an influential factor in

viral detection, in this case especially for the OS, where there is no protocol established for collection. Sample processing problems that influence the presence of proteases and other enzymes may potentiate the destruction of viral RNA before testing (Atkinson 1993; Vosloo 2015). Finally, increasing the frequency of collection could increase the chance of detection and improve IAV monitoring (Gerber 2017; Panyasing 2016).

2.6 Conclusions

This study examined three different sampling methods for the diagnosis of IAV-S in swine, including nasal swabs, oral fluids, and oral swabs. Viral detection was successful in all three types of samples. However, NS showed a better detection rate compared to the other two. For farrowing pigs as well as for nursery pigs, OF demonstrated to be more sensitive than OS.

Results from our study also showed a slight to fair degree of agreement between OS and NS at the individual and within-pen levels, respectively. While this was moderate for the OF when compared to the NS. The degree of agreement between both OF and OS against NS remained the same for farrowing and nursery pigs when these two stages of production were analyzed separately. An excellent degree of agreement with the gold standard sample would mean that the alternative sample tested could hypothetically replace the sample commonly used for surveillance. Thus, surveillance studies of specific pathogens could be improved.

Further research is necessary to accurately evaluate the usefulness of OF and OS for detection, subtyping, and viral isolation. Evaluation of these alternative sampling types in farms with higher viral presence could be beneficial for the development of surveillance protocols. This improves both the quality and subsequent detection rate while making sampling more practical subsequently more beneficial for population surveillance. However, it is also important to validate these sampling techniques on farms with lower virus circulation to replicate common scenarios seen on farms when the initial outbreak and high viral circulation subsides.

2.7 Acknowledgements

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Table 2.1 Monthly samples received per sample type and stage of production.

		Month						
Sample Type	**Stage of production**	**March**	**April**	**May**	**June**	**July**	**September**	**Total**
Nasal Swabs	*Farrowing (sows and piglets)*	18	18	18	18	18	18	108
	Nursery	12	12	12	12	11	12	71
	Total	30	30	30	30	29	30	179
Oral Fluids	*Farrowing (sows only)*	3	3	5	4	4	5	24
	Nursery	6	6	6	6	4	6	34
	Total	9	9	11	10	8	11	58
Oral Swabs	*Farrowing (sows and piglets)*	18	18	18	18	18	18	108
	Nursery	12	12	12	12	12	12	72
	Total	30	30	30	30	30	30	180

Table 2.2 Summary of Real-time PCR results by sample type and category.

	Positive		**Negative**	
Sample type	*Frequency*	*Percent (%)*	*Frequency*	*Percent (%)*
Nasal Swabs	28	15.7	150	84.3
Oral Fluids	10	17.2	48	82.8
Oral Swabs	12	6.7	167	93.3

Table 2.3 Measure of agreement (Kappa), relative sensitivity and specificity of oral fluids and oral swabs at individual- and pen-level compared to nasal swabs.

Stage of production	Sample type	Kappa			Relative sensitivity (%)		Relative specificity (%)	
		Value	95% CI	p	Value	95% CI	Value	95% CI
All	*OF*	0.65	0.41-0.88	0.00	60	59.21-60.79	97.67	97.53-97.82
	OS Individual	0.39	0.19-0.59	0.00	32.14	31.59-32.70	97.99	97.91-98.01
	OS Pen	0.42	0.17-0.67	0.00	35.3	34.57-36.02	98.18	98.01-98.30
Farrowing	*OF (sows)*	0.65	0.36-0.95	0.00	60	59.03-60.97	100	100
	OS Individual	0.35	0.12-0.59	0.00	31.58	30.91-32.25	96.55	96.43-96.67
	OS Pen	0.48	0.16-0.79	0.00	45.45	44.51-46.40	96	95.75-96.23
Nursery	*OF*	0.61	0.21-1.01	0.00	60	58.63-61.37	96.15	95.92-96.39
	OS Individual	0.47	0.12-0.81	0.00	33.3	32.35-34.32	100	100
	OS Pen	0.25	- 0.15-0.65	0.02	16.68	15.71-17.62	100	100

OF: Oral Fluids; OS: Oral Swabs.

Sample Type	Stage of production	Mar	Apr	May	Jun	Jul	Sep
Nasal swabs	*Farrowing*	**NT**		**H3N2**		**H3N2**	**H3N2**
	Nursery	**NT**	**H1N1**			**H3N2**	
Oral fluids	*Farrowing (sows)*					**H3N2**	**H3N2**
	Nursery		**H1N1**				
Oral swabs	*Farrowing*			**H3N2**		**H3N2**	**H3N2**
	Nursery		**H1N1**				

Green: Negative sample; Red: Positive Sample.

NT: Nontypeable

Figure 2.1 Heat map of Matrix RT-qPCR and subtyping results per month, sample type and stage of production. Each box was colored red for positive if one sample per production stage per month was positive, and green if no positive samples were found in the production stage during the same month.

3 SURVEILLANCE OF INFLUENZA VIRUSES IN WESTERN CANADIAN SWINE FARMS AND THEIR WORKERS

This chapter presents the results of a pilot project conducted to investigate associations between influenza A virus in pigs and farmworkers on farms in western Canada. This was an initial assessment to isolate and characterize the viruses circulating in the pigs on these farms over one flu season and determine the feasibility of such a study on a larger scale throughout Canada.

Full citation: Murcia-Rodriguez DM, Harding JCS, Kirychuk S, Detmer SE (2016). Surveillance of influenza viruses in western Canadian swine farms and their workers.

Author Contributions: Detmer SE and Moshynsky I conducted the sample collection and laboratory testing for this study. Murcia DM, Harding JCS, Kirychuk S and Detmer SE contributed to the conception and design of the study, the acquisition, analysis, and interpretation of the data, and the writing of the manuscript.

3.1 Abstract

Influenza A viruses (IAV) that can infect both humans and pigs pose a threat to public health as well as health and economic burdens for pig production. Various studies have shown that the link between swine IAV (IAV-S) and human IAV indicates that swine workers and their families have a greater probability of infection compared to the general population. Therefore, it is important to include swine workers in studies of IAV-S on endemically infected farms. Surveys of clinical symptoms, nasal swabs (NS) and oral swabs (OS) were collected from 26 swine workers (n=130) on 11 farms in Western Canada where 10 pig nasal swabs were being collected for an active surveillance project. The samples collected monthly between October 2015 and May 2016 were tested by matrix RT-qPCR (Ct < 38 considered positive). In total, four samples from the people were found positive; one corresponded to a NS and the remaining three were OS. Two of these human samples were subtyped as H1. Swine NS were pooled and tested. Of the 153 pools tested, 53 (34.64%) pools were positive. From these, 34/53 (64.15%) pools were subtyped, and 27 (79.41%) were classified as H1N2 viruses. While trends were observed, no significant correlation was found between clinical symptoms in humans and RT-qPCR results from swine. In humans, there were significant correlations (Spearman; $P<0.05$) between NS results and cough, as well as NS and sore throat. Pearson correlation examining the pig NS pools and the human Matrix PCR results demonstrated significant correlations between human NS and NS pig pools, as well as human OS and NS pig pools. Even though interspecies transmission was not confirmed in this study, a worker who reported mild influenza-like illness (ILI) symptoms and had a positive level Ct was the only epidemiological link between two farms where an alpha-3 virus moved between pigs at the same time. Further research is needed with improved sample collection methods and an increased sample size to further delineate these potential links. This research may be of importance for the early detection of IAV-S strains with the potential to infect humans. Larger projects involving more swine workers and farms spanning multiple geographic regions are important to assess the prevalence of IAV-S, risk factors for both humans and pigs, and the development of preparedness plans of IAV-S infections in the broader swine and human populations.

3.2 Introduction

Influenza A virus (IAV) is the most commonly detected member of the family *Orthomyxoviridae* across mammals and birds, and many IAVs have zoonotic potential that represents both health and economic threats for humans and animals worldwide (Vincent 2008). IAV in swine (IAV-S) transmission between humans and pigs was first suspected during the 1918 influenza pandemic when respiratory disease outbreaks and pneumonia were observed in both humans and pigs (Paccha 2016b). Since then, periodic zoonotic events between humans and pigs have been confirmed in North America, Europe and Asia (Myers 2007).

Although very limited numbers of these human-to-pig and pig-to-human zoonotic events have resulted in sustained transmission in the aberrant host, the increased pig, and human surveillance in the United States over the last decade has provided insights into cross-species transmission events (Bowman 2014; Nelson 2016; Rajao 2015). While relatively few cases of human influenza were associated with IAV-S between 1958 and 2005 (Myers 2007), surveillance at agricultural fairs and in humans over the last decade has resulted in higher reporting of variant IAV-S strains (Anderson 2015; Feng 2013; Hoa 2015; Kitikoon 2013). Most variant IAV-S infections detected in people have occurred after recent contact with pigs, demonstrating a link between swine exposure and the increased risk of IAV-S infection in humans. Other studies using serological techniques have shown not only that swine exposure enhances the risk of infection with IAV-S, but also that swine workers and their families have a greater probability of getting infected than the rest of the population (Gray 2007a; Olsen 2002b).

The aim of this pilot project was to examine IAV-S at the human-animal interface. This was accomplished by assessing the relationship between clinical symptoms and the presence of IAV-S in swine workers, and by evaluating the viral presence and genetic relatedness of IAV-S present in pigs that have direct contact with the worker population.

3.3 Materials and methods

3.3.1 Ethics statement

The animal sample collection was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use (permit #20120097). The human sample collection was approved by the University of Saskatchewan's Biomedical Research Ethics Board (Bio #14301).

3.3.2 Farm, animal and human subject selection

Eleven IAV-S historically positive farms from Alberta, Saskatchewan, and Manitoba were selected to collect monthly samples (October 2015 to April 2016) from pigs and at least one worker in contact with the pigs. Ten nasal swabs were collected by trained farm personnel from 10 pigs that were 14 to 24 days of age as previously described (Detmer 2013a) using rayon-tipped, culture swabs (Becton, Dickinson, and Company, Mississauga, Ontario, Canada; BD 220099).

At least one person working with the pigs from each of the 11 farms volunteered to participate in the study for a total of 26 swine workers. Each worker was assigned an anonymous 6-digit identifier and a package of instructions before the study started. The package included a video and written instructions on how to collect oral and nasal swabs from themselves and how to pack the samples for shipping to the laboratory at the Western College of Veterinary Medicine, Saskatoon. Each monthly sampling kit contained a survey, and the first survey included further questions indicating general health and work duties as well as an informed consent with information about the study. Monthly surveys ascertained the person's recent presence of influenza health-related symptoms, including headache, muscle pain, fever, runny nose, sore throat, sputum expectoration, and cough. These symptoms were categorized according to a frequency score consisting of 4 categories: never, occasional, often and very often. In addition to the survey, workers collected a nasal swab and an oral swab as previously described (WHO 2011, 2014) using flocked swabs in viral transport media (Puritan Diagnostics, Maine, USA; UT-116 and UT-361). A detailed chart of the participants is shown in **Appendix Table S3.1**.

3.3.3 Testing procedures

3.3.3.1 Sample processing

All swine nasal swabs were cut into a sterile test tube containing 1.5 ml of viral culture media and vortex for 10 s before being transferred to a 2 ml cryovial. The human samples were vortexed for 10 s, and the viral transport media (VTM) was transferred to a 2 ml cryovial. Samples were stored frozen at -80°C.

Pig samples were pooled in three sets (3, 3 and 4 swabs per pool) using equal volumes to total 500 μL of media. Human samples were tested individually using 500 μL of VTM. The

RNA was extracted from the 500 μL of either the sample or pool according to manufacturer instructions using a commercial kit (Qiagen; Maryland, USA; RNeasy Kit 74106).

3.3.3.2 Real-time RT-PCR (RT-qPCR) assays

A commercial RT-qPCR kit (Life Technologies, Texas, USA, 4415200) was used according to manufacturer instructions to detect the IAV matrix gene in 8 μL of extracted RNA from all human and pig samples. For all RNA extracts with a Ct value of 35 and under, a commercial real-time RT-PCR kit (Life Technologies, Texas, USA; 4485541) was used according to manufacturer instructions to detect H1, H3, N1 and N2 genes for subtyping. For both assays, Ct values equal to or less than 38 were positive and values greater than 38 were considered negative. The PCR cut-off values were established and validated by the kit manufacturer and Iowa State University Veterinary Diagnostic Laboratory (Spackman 2014, Zhang and Harmon 2014).

3.3.3.3 Virus isolation and sequencing analysis

Virus isolation was attempted on one positive pool per farm per month at the University of Minnesota Veterinary Diagnostic Laboratory (UMVDL) on swine samples with a Ct value less than 32, as previously described (Detmer 2013b). Viral isolates were forwarded to the J. Craig Venter Institute for whole-genome sequencing and the sequences were published in GenBank.

The evolutionary history of both H1 and H3 lineages was inferred using the Neighbor-Joining method and computed using the Maximum Composite Likelihood method in Mega6 (Saitou and Nei 1987; Tamura 2004; Tamura 2013). The bootstrap test results on 500 replicates are shown as a percentage next to the branches (Felsenstein 1985; Saitou and Nei 1987; Tamura 2004; Tamura 2013).

3.3.4 Statistical analysis

Information from the surveys, as well as Matrix PCR Ct values from both human and swine samples, were compiled, and a descriptive analysis of samples and surveys was performed using IBM SPSS Statistics 23. Scatter plots, Pearson correlation test for two continuous variables and Spearman correlation analysis for non-parametric continuous and categorical variables were performed using IBM SPSS Statistics 23. Variables included in the correlation analyses were worker symptom scores, the Ct values of the human nasal and oral swabs, the Ct values of the three separate pig pools and the average Ct value for the pig pools.

3.4 Results

3.4.1 Human samples and observations

Farms were enrolled between September and January, and their participation was from October 2015 until April 2016. From the 26 human participants who started, only 6 (23.1%) submitted all seven monthly samples and surveys between October 2015 and April 2016. Two (7.7%) participants dropped out of the study after the first sampling. Two months, February and April, had the most participants enrolled with 22 (84.6%) individuals enrolled in each of these months (**Appendix Table S3.2**).

The most common symptoms of ILI in humans (headache, muscular pain, fever, runny nose, sore throat, sputum, and cough) were evaluated, and their frequency was analyzed. Across all the symptoms 'Never' and 'Occasional' were most commonly reported varying from 26 (20.6%) to 84 (67.2%) times reported, respectively. Muscle pain was most frequently reported as occurring 'very often' with 12 (9.5%) reports, followed by sputum, scratchy throat and cough with 10 (8.1%), 7 (7%) and 7 (5.7%) reports, respectively. The frequency and distribution of clinical symptoms had minimal variation between subjects (**Appendix Table S3.3**).

3.4.1.1 Real-time RT-PCR assay

From the 26 participants, 130 (71.4%) nasal swabs and 130 (71.4%) oral swabs were received out of the anticipated 182 nasal swabs and 182 oral swabs. The analysis was based on the total samples received. Matrix gene PCR assay results from nasal swabs revealed 129 (99.23%) negative and 1 (0.77%) positive samples. Similarly, the oral swabs revealed 127 (97.69%) negative and 3 (2.31%) positive samples (**Appendix Table S3.4**).

3.4.1.2 Human Subtyping analysis

Two (66.6%) of the three positive oral swab samples were subtyped and classified as H1 viruses, whereas the positive NS failed to be classified by RT-PCR (**Appendix Table S3.5**).

3.4.1.3 Correlation analysis

Each of the ILI human symptoms, from all individuals, were compared with the IAV-S Matrix PCR Ct results from all human nasal and oral swabs. Spearman correlation analysis between the symptom scores and the Ct values revealed a weak significant correlation with cough (rho=0.21 p=0.03) and sore throat (rho=0.18 p=0.05), the remaining clinical symptoms showed no correlation with the human RT-PCR results (**Table 3.1**).

3.4.2 Pig samples and Matrix PCR results

All pig samples were received with at least one set of human samples and a symptom survey. Two farms (18.18%) out of the 11 farms tested had no PCR positive pig pools throughout the study period, indicating that no virus was detected during the study period in pigs pre-weaning. Four farms (36.36% of the farms) were positive during more than three months of the study, while the remaining five (45.45%) had lower viral detection, being positive only in one or two months of the study. One of the farms showed a continuous presence of IAV during the months samples were received (**Figure 3.1**).

Of the 153 pools tested, 53 (34.64%) were positive, and 100 (65.36%) were negative. Farms were more frequently positive for IAV-S in January and February with six positive farms (54.54%) for each of these months, while October and March were the months with fewer IAV-S positive farms. The rest of the months had between three and five positive farms (**Figure 3.1**).

Pearson correlation analysis of all PCR results between the three swine pools was positive and had a strong correlation (r=0.638 to 0.789 p=<0.001) (**Appendix Table S3.5**). Correlation analysis of the three swine pools against the average pool Ct score revealed a very strong correlation (r=0.896 to 0.923 p=<0.001) (**Appendix Table S3.6**).

3.4.2.1 Subtyping analysis in pig populations

From the 53 positive pools, 34 (64.15%) were subtyped. The most common subtype found among the 11 farms during the time frame of the study was H1N2 with 27/34 (79.41%). Other subtypes were found with less frequency including one H1N1 (2.94%) (**Figure 3.1** and **Appendix Table S3.7**).

3.4.2.2 Virus isolation and sequencing analysis

One H1N1, 27 H1N2, and 4 H3N2 viruses were isolated. From these isolates, 17 H1 and 13 H3 sequences were obtained. The H1 sequence analysis involved 30 nucleotide sequences and a total of 1692 nucleotide positions in the final dataset (**Figure 3.2**). During the 2015-16 season, the sequence from farm 3 was in the 2009 pandemic H1N1 clade (1A.3.3.2), and sequences from farms 1, 4, 5, 9 and 10 were from the swine H1 alpha clade (1A.1.1). Historical sequences from farms 3 and 9, and farms 1, 2 and 3 were from the 2009 pandemic H1N1 and swine H1 alpha clades, respectively. The H3 sequence analysis involved 25 nucleotide sequences and a total of 1699 positions in the final dataset (**Figure 3.3**). During the 2015-16 season, three sequences

from farm 8 were H3N2 clade IVC-like viruses. Ten historical sequences from farms 1 and 2 were H3N2 clade IV viruses.

3.4.3 Interspecies analysis

Correlation analysis was performed that included Ct values from all human nasal swabs and oral swabs, symptom scores, and swine nasal swabs. Matrix PCR results from human nasal and oral swabs had a very weak correlation with the average Ct values of the swine pools (r=0.20 p=0.03; r=0.18 p=0.05. respectively). When comparing the swine sample results to the symptom scores, there was no correlation between any of the clinical symptoms evaluated and the swine Ct values (**Table 3.1**).

Even though the interspecies transmission was not confirmed, in 2 (18.2%) of the 11 farms, humans had Ct values within the positive range at the same time that swine pools were positive. On one of the farms, two swine workers were positive while the swine pools were positive as well (**Figure 3.4**).

3.5 Discussion

In this study, the linkage of IAV-S was examined at the human-porcine interface on 11 conveniently selected farms located in Western Canada. Within the seven months study duration, no evidence of transmission between swine workers and pigs was detected. Humans enrolled in the study reported infrequent ILI symptoms. Workers who had positive samples reported never to occasional presentation of the symptoms evaluated. Meanwhile, influenza activity in pigs varied from absent (no detection) on two farms to the detection of several positive pools, during several months of the study. Positive samples were observed to occur primarily in January and April, and with a higher presence of the H1N2 subtype.

Sporadic reports of IAV-S transmission between swine and people (Myers 2007) highlight the importance of studying relationships between human clinical signs and detection of virus in pigs. In this study, there was no significant correlation between clinical symptoms in people and swine Ct values. However, matrix PCR results from human nasal and oral swabs had a weak correlation with the average Ct values of the swine pools (r=0.20 p=0.03; r=0.18 p=0.05, respectively). Even though the interspecies transmission was not confirmed, positive human results coincided with positive swine pools on 2 of 11 farms, and these farms had the Alpha-3 H1N2 which was detected in a human in May 2016 (Budd 2016). These results highlight the variation in clinical symptoms and the overall infrequency of symptoms being present and are

consistent with studies that show that IAV-S transmission between humans and swine occurs at a very low rate (Olson 1977; Van Reeth 2007).

The occurrence of influenza in people is usually evaluated based on reports of ILI activity and viral detection. Public Health Agency of Canada reported an ILI prevalence between 1.25% (12.5 cases per 1000 patients) and 7.56 (75.6 cases per 1000 patients) during the 2015-16 seasonal flu season (PHAC 2017). This behavior was more extreme than one of the previous seasons where the prevalence was estimated to be between 2.31% (23.14 cases per 1000 patients) and 4.99% (49.9 cases per 1000 patients) (PHAC 2017). In this study, the clinical symptoms were infrequently reported among the swine workers. However, muscle pain, sputum, scratchy throat, and cough were reported as present 'very often' but only by a few of the human subjects over occasional months. These results are similar to studies showing the mild clinical presentation (presence of ILI symptoms) of IAV-S in humans (Gray 2007b).

The low rate of detection in human samples made an accurate analysis of the prevalence of IAV-S in people difficult. Although the collected samples included both human nasal swabs and oral swabs, which have been reported as preferable samples for the diagnosis of viral infections of the upper respiratory tract by the World Health Organization (WHO 2011), the lack of viral detection in the swine workers might be related to the collection technique and timing of sampling. In the present study, nasal swabs and oral swabs were taken directly by the person participating in the experiment without previous formal training. The lack of experience, as well as the discomfort of sample collection, may have affected the quality of the samples. Also, for the nasal swabs, only one nostril was sampled rather than both nostrils, as recommended (WHO 2011).

Samples were collected once a month during the 2015-16 flu season. Extra kits for sample collection were provided to participants with instructions to collect a sample if they were experiencing flu-like symptoms. None of the kits were used by the participants even if they reported experiencing clinical symptoms before the monthly collection. Reports show that the incubation period for flu in humans ranges from one to four days, with a peak viral shedding one day before the onset of the clinical symptoms and up to three days after clinical symptoms started (Shinde 2009; Zambon 2001). Peak shedding can also coincide with a fever in humans. For these reasons, it is recommended that respiratory specimens be taken within three days of the onset of clinical symptoms (WHO 2011). Human samples were collected on a set day together

with pig samples, regardless of clinical symptoms in either species. Both the low frequency of sampling and the non-usage of the extra kits when people were reportedly ill could account for much of the limited detection of IAV in this study.

Even though the inclusion criteria for the selection of the farms was a recently reported presence of IAV-S in pigs, within the period of study no virus was detected on two of the farms. In the rest of the farms, positive results were found in one to six months of the study. Results may have also been affected by the different time frames of participation among the 11 farms. Even though the study was undertaken during the period of higher seasonal activity for influenza viruses (October to May), the previous flu season (2014-15) had greater seasonal influenza activity. Comparing the results of all samples submitted to our lab from the 2015-16 season to the results from 2014-15, 22.7% of the samples were positive compared to 36.3% previously (Unpublished data).

The subtyping analysis from both the positive and the suspect pig samples showed that the most common subtype present was H1N2. Sequencing revealed that these are in the new alpha-3 subclade of the swine H1 alpha clade present in American and Canadian pig populations (Nelson 2017). Before the 2015-16 season, there was a more even spread of Clade IV H3N2, 2009 pandemic H1N1 and swine H1 alphaviruses within western Canada (Nelson 2017). However, during the 2015-16 season, there was an upsurge of alpha-3 virus detected in western Canada by our laboratory (unpublished data). However, further studies are required to compare our results with the prevalence and subtype distribution of IAV-S in the swine industry across Canada.

3.6 Conclusions

This study examined IAV-S at the human-swine interface by evaluating both clinical signs and viral detection. Even though zoonotic transmission was not confirmed, multiple events where the virus was present in both human and swine on the same farm and at the same time infer a link between humans and swine which needs to be examined further and demonstrates the importance of active surveillance in both humans and swine. One such event involved a sample with a positive level Ct collected from a human with mild ILI which was the only epidemiological link between two farms where an alpha-3 virus moved between pigs **(Figure 1)**. This resulted in a new introduction of alpha-3 from Manitoba pigs to a farm in Saskatchewan.

Results from this study also highlight the variation in clinical symptoms in humans and the overall infrequency of symptoms being present, as well as the importance of sample timing for detecting influenza viruses. Larger projects involving more swine workers and farms spanning multiple geographic regions are important when looking at the prevalence of IAV-S, risk factors for both humans and pigs, and the early development of preparedness plans of IAV-S infections in the broader human population.

3.7 Acknowledgements

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Table 3.1 Pearson and Spearman Correlation results of all human symptom scores and Ct values of all Matrix PCR results from human and swine samples.

	Human NSW		**Human OSW**		**Pig Pool Average**	
Variable	*R*	*p*	*R*	*p*	*R*	*p*
Human NSW*	1	-	0.07	0.45	**0.20**	**0.03‡**
Human OSW*	0.07	0.45	1	-	**0.18**	**0.05‡**
Headache†	0.10	0.32	0.02	0.83	-0.02	0.87
Muscle†	-0.06	0.53	0.14	0.15	-0.05	0.64
Fever†	0.11	0.24	0.06	0.53	0.04	0.65
Runny nose†	0.02	0.87	0.01	0.89	-0.01	0.95
Sore throat†	**0.18**	**0.05‡**	0.01	0.89	-0.01	0.95
Sputum†	0.18	0.06	0.05	0.62	0.08	0.39
Cough†	**0.21**	**0.03‡**	0.07	0.46	0.09	0.36
Pig pool average*	**0.20**	**0.03‡**	**0.18**	**0.05‡**	1	-

* Pearson Correlation test

† Spearman Correlation test

‡ Significant correlation at the level of P=0.05 (bilateral).

Farm	Oct	Nov	Dec	Jan	Feb	Mar	Apr
1	NT		H1N2	H1N2	H1N2		H1N2
2		H1N1		H1		H1N2	H1N2
3							
4		H1N2	H1N2	H1N2	H1N2	H1N2	H1N2
5		H1N2					
6					NT		
7							
8			H3	H3	NT		H3
9				H1N2	H1N2		
10					H1N2		H1N2
11				NT			

Green: Negative sample; Red: Positive Sample; White: No sample.

NT: Not typeable

Figure 3.1 Heat map of swine Matrix RT-qPCR and subtyping results per month per farm. Each box was colored red for positive if one sample per month was positive, white if not samples were submitted that month, and green if no positive samples were found during the indicated month in a particular farm.

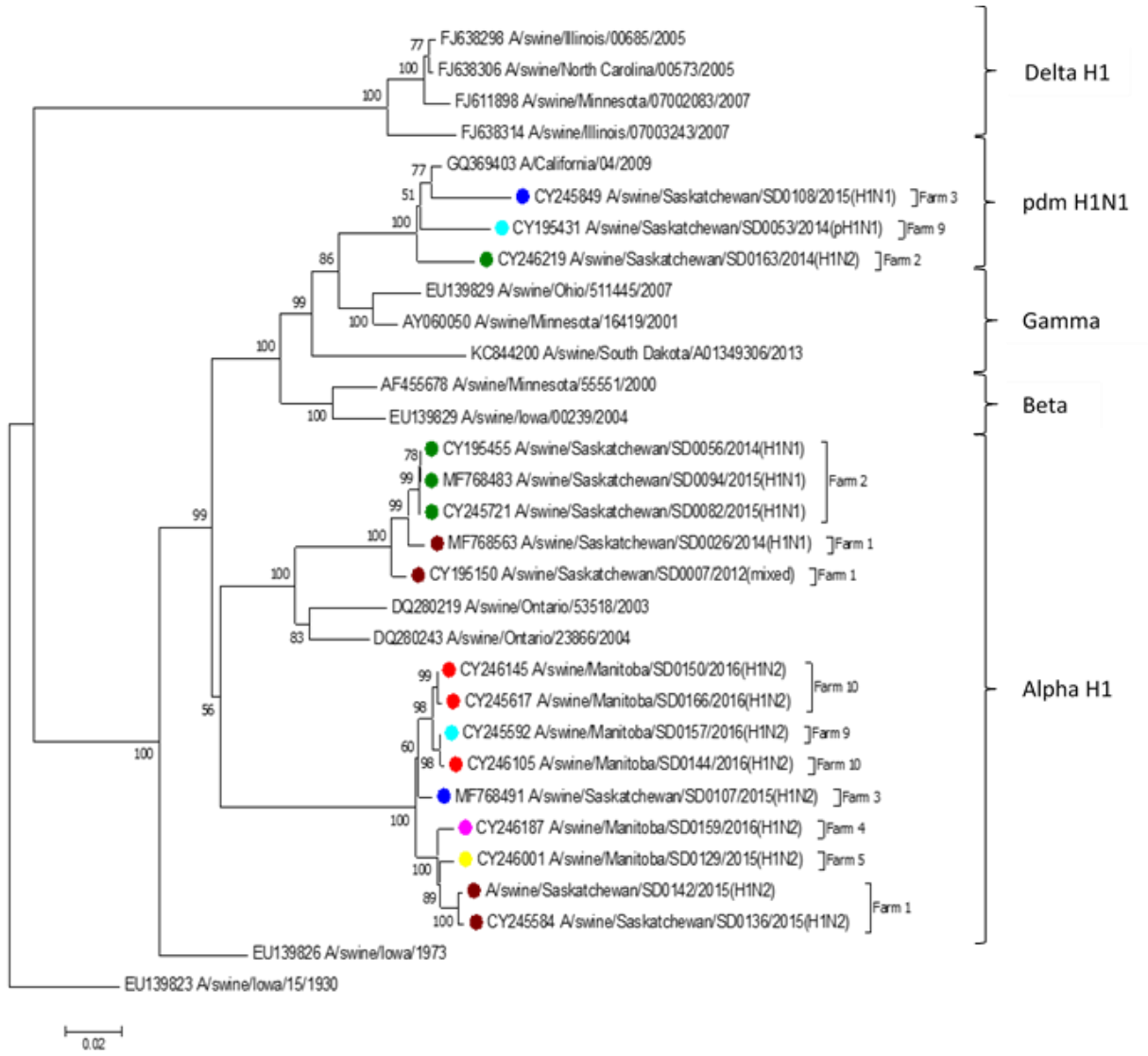


Figure 3.2 Evolutionary relationships of H1 from project farms. The evolutionary history was inferred using the Neighbor-Joining method (Saitou 1987), and the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura 2004). Farms 1 and 10 show minimal evolution during the study period. Farms 9 and 10 are unrelated sites in the same region having the same virus during the study period. A different color dot was used for each farm in the study.

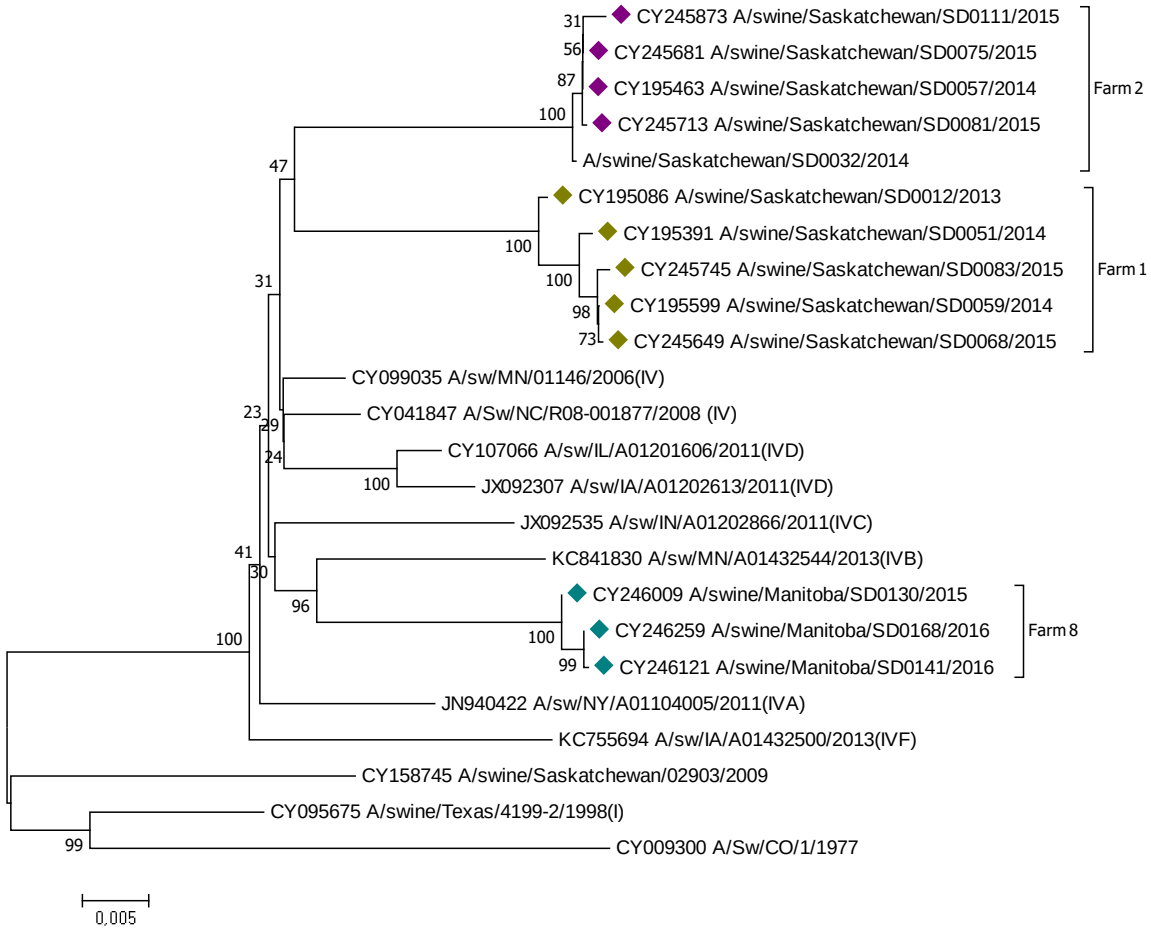


Figure 3.3 Evolutionary relationships of H3 from project farms. The evolutionary history was inferred using the Neighbor-Joining method (Saitou 1987), and the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura 2004). Three isolated sequences from farm 8 demonstrated evolution during the study. Different colors indicate the different farms.

Farm	Type of sample	Oct	Nov	Dec	Jan	Feb	Mar	Apr
1	Human 1							
	Human 2							
	Human 3							
	Human 4							
	Human 5							
	Average Pig Pool 1							
2	Human 6							
	Human 7							
	Average Pig Pool 2							
3	Human 8							
	Human 9							
	Human 10							
	Average Pig Pool 3							
4	Human 11							
	Human 12							
	Human 13							
	Human 14							
	Average Pig Pool 4							
5	Human 15							
	Human 16							
	Human 17							
	Human 18							
	Average Pig Pool 5							
6	Human 19							
	Human 20							
	Average Pig Pool 6							
7	Human 21							
	Average Pig Pool 7							
8	Human 22							
	Average Pig Pool 8							
9	Human 23							
	Average Pig Pool 9							
10	Human 24							
	Average Pig Pool 10							
11	Human 25							
	Human 26							
	Average Pig Pool 11							

Green: Negative sample; Red: Positive Sample; White: No sample.

Figure 3.4 Heat map showing the Matrix RT-qPCR results from human and swine samples per month. A month result was defined as positive (red) if one sample per farm per month was positive, and as negative (green) if no positive samples were found during the indicated month in a particular farm.

4 GENERAL DISCUSSION

4.1 Discussion

Influenza A viruses (IAVs), the predominant type of influenza virus detected in mammals and birds and is one of the most recognized zoonotic viral pathogens. IAV represents economic and health threats to both animals and humans worldwide (Vincent 2008). Successful monitoring and surveillance programs should provide data necessary for the better understanding of the determinants of infection to develop better disease control and prevention plans (Corzo 2013), and this thesis provides a starting point for larger-scaled human-swine surveillance.

In humans, surveillance programs for IAV are highly organized at the national and international levels. In comparison, animal surveillance for IAV is often spotty and poorly funded (Vincent 2014). Multiple laboratories under the direction of the WHO established as primary objectives the provision of detailed virological information about influenza viruses in humans and monitoring of novel influenza viruses that can pose a risk to communities and may cause a pandemic (WHO 2011). However, one of the main gaps of the WHO plans for surveillance of IAV is not including swine workers as a specific monitoring population, even though this particular group has been established to be at high risk for IAV infection (Gray 2007b; Myers 2006; Olsen 2002a). Swine workers are also considered to serve as a source of infection for the community and other species (Gray 2007b).

Animal surveillance for IAV is entirely different from that of humans. Surveillance data can be limited in some countries due to limited funding, a lack of centralized organization or communication structure, and less technical resources. The lack of support for animal influenza may be due to the fact that IAV has not been recognized as a notifiable disease by the OIE. Other reasons include that there are limited resources for animal surveillance for all diseases, the low impact of IAV in some countries on animal health and productivity, the fear of negative trading consequences, and the fact that increased movement of pigs and humans around the globe requires international cooperation to implement a global animal surveillance program (Detmer 2013a).

The gaps observed in the surveillance of IAV in swine workers as well as the low rate of implementation of it in the animal population, make a global animal-human surveillance system difficult. For timely identification and detection of circulating IAV of important to both human and animal health, coordination between human and animal agencies to consolidate and compare

data across the two sectors should be a central goal of government and international health organizations. In the present study, active surveillance of both human and swine populations was performed.

In swine populations, monitoring programs of IAV-S have used nasal swabs (NS) as their gold standard sampling method (Corzo 2013). The costs associated with sample collection (labor, supplies and time) plus the testing costs mean that this may not be the most economical method to find IAV-S on a farm (Panyasing 2016). Thus, alternative sampling methods like oral fluids, oral swabs, snout wipes, udder wipes, and air samples have been explored because they require less or no animal restraint and that test more animals with a single sample.

In chapter 2, a comparison between NS and two alternative samples were examined, oral fluids (OF) and oral swabs (OS). The Matrix gene PCR results confirmed that NS was the best sample for detecting IAV-S on the study farm compared to the other two sample types. Thus, the sensitivity and ability of the sample to detect the virus need to be part of the economic assessment. It is important to consider that a farmer may not save money in the long run if infections go undetected.

Despite the relative ease in the collection of OF and OS compared to the NS, the relative sensitivities of both were found to be only between 32.14% and 60%. These findings differ from reports showing an overall sensitivity above 80% for OF (Detmer 2011; Romagosa 2012) and showing OS as the best individual sample for detection of IAV-S (Mantilla Garrido 2017). Furthermore, this study highlights the effectiveness of OF in the two stages of production evaluated (farrowing and nursery). In the present study, the most efficient sample type during the farrowing stage after the NS was found to be the OF. These results are similar to previous reports showing OF as a better sample during this stage (Strobel 2016).

However, it is important to recognize that in the present study, OF from this stage of production were collected from the individual sows given the difficulty of collection in younger pigs (CFSPH 2015; Hernandez-Garcia 2017). Similarly in nursery pigs, OF were more efficient than OS. The age-specific behavior of this group allowed the collection of OF to happen in all the animals present in the pen selected. Results from this study showed that OF had a moderate degree of agreement when compared to NS when analyzing them as a whole population as well as within the two stages of production evaluated in the study. These findings differ from other reports an excellent agreement between NS and OF (Romagosa 2012). In contrast, OS showed a

slight to fair agreement with NS. These findings are similar to other reports showing OS to have a fair degree of agreement (Strobel 2016).

Surveillance of IAV-S on the selected farm showed that the two most common subtypes present were H1N1 and H1N2, but introductions of an H3N2 virus was also observed in a farm with the previous history of H1N1 presence. These findings are consistent with other studies showing the presence of both H1N1 and H3N2 viruses in western Canada (Nelson 2017). Nelson (2017) reported an even spread of Clade IV H3N2, 2009 pandemic H1N1 and Alpha H1N1 viruses within west Canada before the 2015-16 season, as well as an upsurge of the Alpha H1N2 virus in the region within the same season. This information may be a key to continual development and improvement of IAV-S surveillance in Canada. The inclusion of more farms and the ability to provide high-quality data can serve as a primary mechanism for decision-making involving all the stakeholders and including areas such as management, reporting, and prevention of important zoonotic pathogens as IAV.

The occurrence of influenza in people in this thesis was evaluated based on the presence of symptoms related to flu and the Matrix gene PCR results from nasal and oral swabs from swine workers. In chapter 3, influenza-related symptoms were mild and infrequent, similar to other studies showing mild clinical presentation of IAV-S in humans (Gray 2007b). The low rate of detection in human samples made an accurate analysis of the prevalence of IAV-S in people difficult.

The behavior of IAV-S was also examined at the human-animal interface in the selected farms of Western Canada. Multiple reports of IAV-S transmission between humans and pigs have highlighted the importance of studying the clinical presentation of flu in humans while undergoing detection of influenza in swine. In this study, there was no confirmation of transmission among species, and there was no significant correlation between the clinical signs evaluated in the swine workers and the Matrix gene PCR results from the NS from pigs. However, results from the NS and OS of these people had a very weak correlation with the Matrix gene PCR results from swine. Also, in two of the farms studied humans had positive results at the same time there were positive swine pools, and all of these farms had the Alpha H1N2 which was reported in a human case (Budd 2016; Resende 2017). These results emphasize the infrequency and variation in clinical signs as well as the overall low rate of transmission between humans and swine, as shown in other studies (Olson 1977). These observations also

show the lack of surveillance available for an important zoonotic pathogen as is IAV, and highlight the necessity for the implementation of monitoring and prevention plans for the control of the transmission between these two species.

Different molecular methods for the diagnosis of IAV are currently available, some of them showing occasional interpretation differences, in terms of Ct value cut-offs. For instance, the diagnostic protocol provided by the National Veterinary Services Laboratories (USDA) in 2012 states that "the Ct should be located in the exponential phase of a 'normal' amplification curve," and "that weak positive specimens with a low level of amplification will be interpreted as negative and no Ct value will be reported." This leaves the exact cut-off values up to the individual laboratories within the National Animal Health Laboratory Network.

One such laboratory UMVDL, classifies the RT-qPCR results into three categories according to the Ct value based on their in-house validation of the assay with NS, OF and lung tissue samples. At this laboratory, samples with Ct<35 are classified as positive (Ct<35), Ct≥40 are negative, and Ct values between 35 and 40 are considered "suspect" and should be retested to confirm. The USDA-licensed assay used in this thesis has only three results: Ct<38 is positive, no Ct detected is negative and Ct 38-40 is a suspect result (Applied Biosystems, 2011). Suspect results for this protocol are retested and most often found to be negative. In addition to the validation of the assay by Life Technologies (now owned by Fischer Scientific), this kit has been used extensively by the Iowa State University Veterinary Diagnostic Laboratory with the 38 Ct cut-off between positive and negative (Zhang and Harmon 2014).

The Centers for Disease Control and Prevention (CDC) in their IAV testing use a similar interpretation, where samples are positive if Ct<38 and negative if they are Ct>38 (Shu 2011). Meanwhile, the Canadian Food Inspection Agency (CFIA) 2014 protocol defines a positive as less than 35.99, no Ct detected is negative and Ct > 35.99 is a suspect result. Prior to 2009, the CFIA used a modified Spackman protocol which classified the positive samples the ones with Ct>33, the negative the ones with Ct<36 and indeterminate samples the ones with Ct values between 33 and 36 (Slomka 2007; 2010). It is important to clarify that each of these different kits or protocols with their Ct cut-offs use very similar primers and probes, have been previously validated, and use either 40 or 45 total cycles.

In this thesis, the results from the Matrix PCR from both humans and pigs were analyzed using two different Ct cut-off values. Initially, the analysis was done with three categories used

by UMVDL which classified the samples into three categories: positive (Ct<35), negative (Ct≥40) and suspect (Ct 35-40) samples. The second cut-off classified samples only into two categories; positive (Ct<38) and negative (Ct≥38). In this second classification, the suspect category is not considered and therefore provided clear results for the statistical analysis and different conclusions of both chapters.

In chapter 2, the use of both cut-offs showed NS as the best method for detection of IAV-S in swine. However, when using the Ct<38 cut-off, the evaluation of the whole population showed an improvement in the OF's relative sensitivity, specificity, and the kappa value. The opposite happened with the OS which had a decrease in the efficiency in terms of lower values for the relative sensitivity, specificity and level of agreement compared to the results observed with the first cut-off ranges. Furthermore, when analyzing the different stages of production there were also differences between the methods. Initially, for farrowing pigs, the best sample for IAV-S diagnosis was pen-based OS, but after undergoing the analysis with the Ct<38 cut-off, OF were found to be better. For nursery pigs, OF were the best sample after the NS using both cut-off value classifications (**Table 4.1**).

Chapter 3 also showed differences when comparing the two cut-off value interpretations. The initial examination of the results for the human samples showed no positive results but only suspect samples. This changed substantially with the second analysis, as the samples switch from 11 suspect samples to 4 positive samples. Similarly, the number of swine positive samples increased showing a different behavior of IAV-S among the different farms (**Figure 4.1**). Additionally, the correlation analysis showed important variations. When using the first method, no correlation was observed between human RT-qPCR results and their clinical symptoms, but when using the second method weak correlation was found between human NS and sore throat, and human NS and cough. Surprisingly, the interspecies correlation analysis remained the same, showing a weak correlation between the human Matrix PCR results and the average Ct values from the pig samples (**Table 4.2**).

4.2 Limitations

In chapter 2, circumstances such as the amount of virus present on the farm, the collection technique, the sample processing and frequency of collection have significant implications in viral detection and probably influenced the results of the study. Other conditions

such as sample size and frequency and timing of sampling during the research may have also affected the results observed.

For both chapter 2 and 3, low amounts of the virus were detected which might have influenced RNA recovery and subsequent low detection (Vosloo 2015). The number of positive samples in both humans and swine in this study, as well as the multiple farms with low to no IAV-S detection, might suggest a lower presence of virus than expected. The unknown effects of pooling the samples could have some implications for the viral detection as well. Furthermore, the amount of virus present in the farms of the study also had repercussions for the rate of virus isolation of IAV-S in swine. Additionally, the low viral presence can also be affected by seasonal changes in IAV pathogenesis and virulence as well as outdoor weather.

Influenza activity was lower in 2015-16 season compared to the results submitted to our lab from the previous 2014-15 season. During the 2015-16 season, 22.7% of the samples were positive compared to 36.3% from 2014-15 (Unpublished data). This lower activity and presence of the virus might have had an association with the detection of IAV, as well as with detection of interface transmission between human and pigs.

Collection technique might have influenced the viral detection for IAV in this study. For chapter 2, the method for collection of OS in swine might explain the differences with other studies, as there is no protocol established for the collection of this type of sample. For the human sample collection in chapter 3, no training was provided to the workers other than a video sent to the participants. Lack of training for the swine workers, as well as the discomfort experienced during collection, may have impacted sample quality. No samples were submitted outside of the monthly collection periods, and therefore, the time of active human influenza may not have been captured.

During sample processing, the presence of proteases and other enzymes may potentiate the destruction of viral RNA before testing (Atkinson 1993; Vosloo 2015) and can make viral detection difficult. In chapter 2, there were over 60 samples collected by a team of two veterinarians in one day. It is possible that the earliest collected samples sat at room temperature while the rest of the samples were collected. This farm was only three hours away from the laboratory. Therefore the samples were received less than 24 hours after collection. Although they were processed the same as other samples in the laboratory, the sheer number of samples may have allowed some samples to sit at room temperature longer than normal. In chapter 3,

human samples were handled in the laboratory similarly to swine samples within the biosafety cabinet and samples were immediately frozen at -80C to protect them from degradation. The 'on farm' sample handling may have differed between humans and pigs, particularly on farms where more than one person participated.

Another significant limitation in chapter 3 was the small sample size used for human IAV-S surveillance. The difficulty of enrolling participants in the study, having participants successfully participate during the whole study time frame, along with the staggered start time for farms created additional complications and limitations. Collecting samples from both humans and pigs each month at a set time regardless of clinical signs and the no use of the extra kits by the swine workers could account for much of the limited detection of infected humans and pigs in this study.

4.3 Future directions

The results of the research presented in this thesis emphasize the importance of surveillance in both human and swine populations and provide a better understanding of IAV-S in Canada. Further research with improved approaches including; inclusion of multiple geographic regions, increased sample size, refined sample collection methods, and additional farm selection criteria would enhance these findings.

Larger projects involving farms from multiple regions in Canada are necessary to examine the prevalence of IAV-S in Canadian swine workers and pigs. Additionally, a broader geographical inclusion might provide critical information for improved insight into epidemiological patterns, risk factors, the burden of disease, the emergence of novel viruses, and human-animal interface transmission. Considering the results laid out in chapter 3, the baseline observations of this study may be useful to predict the sample size for a Canada-wide study.

Increased enrollment of swine workers will allow a better comprehension of the human-animal interface IAV-S situation. More swine worker's participation together with ILI symptoms evaluation and symptom-match sampling collection will provide valuable information for the implementation of pandemic preparedness plans in the broader human population. Especially considering the wide-ranging impacts of the human 2009 H1N1 pandemic and the resulting spread of the pandemic virus to and within the North American swine herd (Nelson 2015a).

Additional work is also needed to look into new alternative samples for IAV-S on farms. Even though the results in chapter 2 continue to show that nasal swabs are the most reliable tool

for the detection of IAV-S in swine, further research is needed. Oral fluids continued to be a promising method (Kulanayake 2015) but viral detection in this study was lower in OF than with NS, and virus isolation success is still limited (Detmer 2011). Oral swabs have been considered an alternative but recommended for other monitoring of other diseases in pigs. However, the lack of a standardized collection protocol and the few studies looking at its usefulness for IAV detection on farms with high IAV-S prevalence (Mantilla Garrido 2017) make further investigation recommended.

Two other samples that warrant further investigation which are already being used on farms are snout wipes and udder wipes. Both have been reported to have high detection and virus isolation rates (Bowman 2014; Mantilla Garrido 2017) for surveillance work. These samples are of interest to farmers and veterinarians for regular disease monitoring programs. To pursue using these techniques, standard procedures need to be developed for sample collection and processing in the laboratory, along with validation of the samples for RT-qPCR to determine if pooling impacts sensitivity.

It will be critical to determine the detection limits of these alternative methods to be able to provide better recommendations that include the use of the most simple, efficient, inexpensive and reliable sample. Furthermore, shorter time intervals between samplings (e.g., two weeks instead of a month) may improve detection rates for comparing alternative samples to NS. This along with selection of farms with an optimal IAV-S load may create more favorable conditions for viral isolation. Having a higher quantity of virus in the samples also may be helpful for epidemiological and viral characterization if it produces higher isolation and sequencing rates.

4.4 Concluding remarks

Influenza surveillance in pigs and people provides critical information in understanding the ecology and evolution of the virus in two of its natural hosts who share influenza virus strains, similar seasonal spikes in disease, and similar progression of clinical disease. Furthermore, the close connection of these two species at the human-animal-environment interface means that influenza surveillance in both species at locations where that interface occurs (e.g., farms, abattoirs, and sale barns) can provide a better understanding of the infection and the usefulness of control measures. The research presented in this thesis provides a glimpse into a broader area of research which should be explored further.

Table 4.1 Comparison between analysis of kappa value, relative sensitivity and relative specificity in oral fluids and oral swabs for two different interpretations of Ct values.

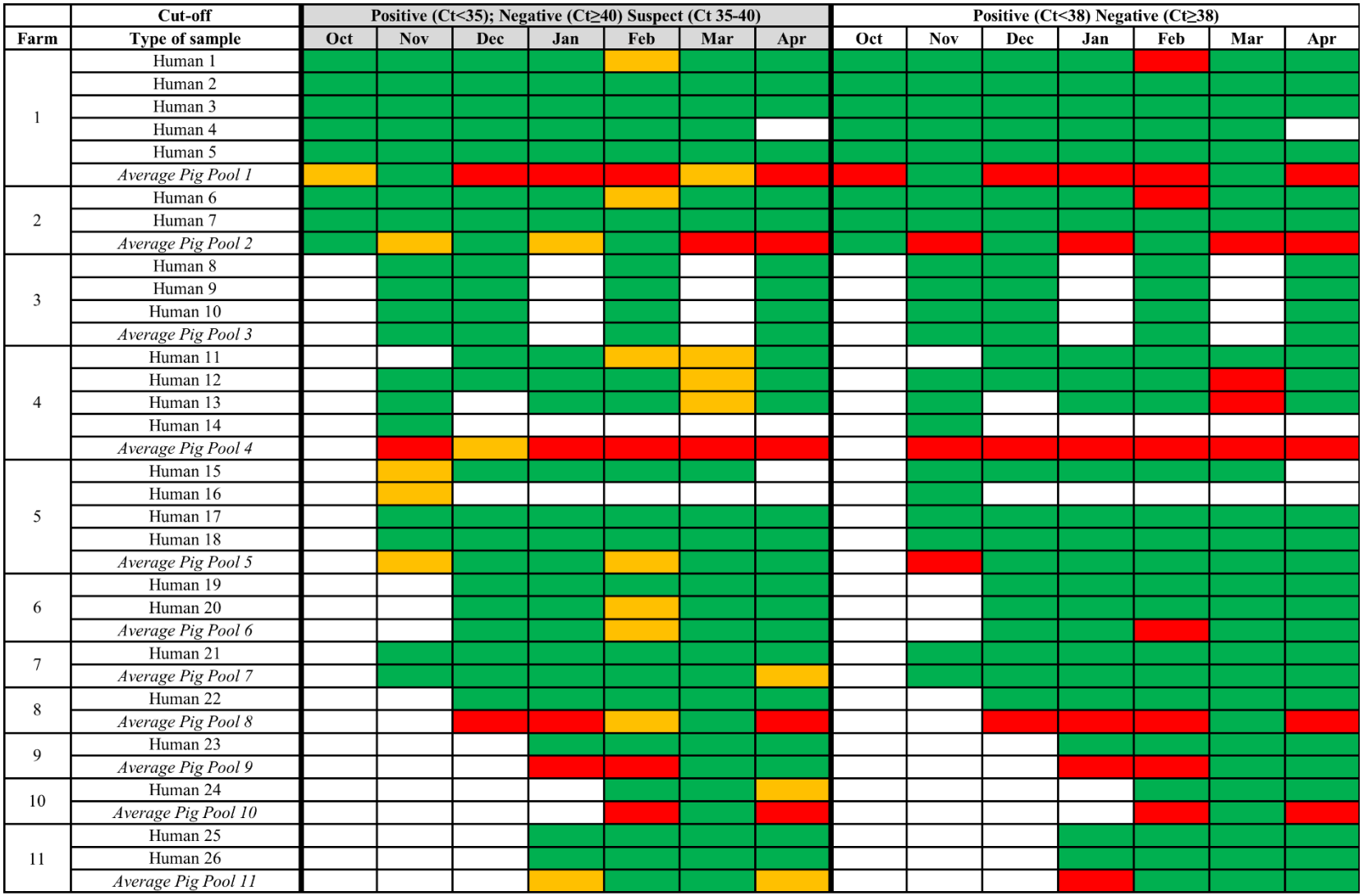
Interpretation (Ct cut-off values)	Stage of production	Sample type	Kappa			Relative sensitivity (%)		Relative specificity (%)	
			Value	95% CI	p	Value	95% CI	Value	95% CI
Positive (Ct<35) Negative (Ct≥40) Suspect (Ct 35-40)	All	*OF*	0.58	0.33-0.84	0.00	57.1	26.4-87.8	95.5	89.6-100
		OS Individual	0.53	0.37-0.69	0.00	43.6	20.7-66.5	99.3	98.0-100
		OS Pen	0.55	0.30-0.79	0.00	43.8	7.04-80.5	100	100
	Farrowing	*OF*	0.65	0.36-0.95	0.00	55.6	15.8-95.4	94.4	84.5-100
		OS Individual	0.35	0.12-0.59	0.00	50	0.25-0.75	98.7	96.3-100
		OS Pen	0.48	0.16-0.79	0.00	60	0.21-0.99	100	100
	Nursery	*OF*	0.61	0.21-1.01	0.00	60	12.0-100	96.2	89.0-100
		OS Individual	0.47	0.12-0.81	0.00	27.3	-0.23-0.78	100	100
		OS Pen	0.25	- 0.15-0.65	0.02	16.7	-0.56-0.90	100	100
Positive (Ct<38) Negative (Ct≥38)	All	*OF*	0.65	0.41-0.88	0.00	60	59.21-60.79	97.67	97.53-97.82
		OS Individual	0.39	0.19-0.59	0.00	32.14	31.59-32.70	97.99	97.91-98.01
		OS Pen	0.42	0.17-0.67	0.00	35.3	34.57-36.02	98.18	98.01-98.30
	Farrowing	*OF*	0.65	0.36-0.95	0.00	60	59.03-60.97	100	100
		OS Individual	0.35	0.12-0.59	0.00	31.58	30.91-32.25	96.55	96.43-96.67
		OS Pen	0.48	0.16-0.79	0.00	45.45	44.51-46.40	96	95.75-96.23
	Nursery	*OF*	0.61	0.21-1.01	0.00	60	58.63-61.37	96.15	95.92-96.39
		OS Individual	0.47	0.12-0.81	0.00	33.3	32.35-34.32	100	100
		OS Pen	0.25	- 0.15-0.65	0.02	16.68	15.71-17.62	100	100

OF: Oral Fluids; OS: Oral Swabs

Table 4.2 Comparison between two cut-offs for the correlation analysis of human Matrix PCR results and clinical symptoms, and swine Matrix PCR results.

Cut-off values	**Positive (Ct<35); Negative (Ct≥40) Suspect (Ct 35-40)**						**Positive (Ct<38) Negative (Ct≥38)**					
	Human NSW		**Human NSW**		**Pig Pool Average**		**Human NSW**		**Human NSW**		**Pig Pool Average**	
Variable	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*	*R*	*p*
Human NSW*	1	-	0.07	0.42	**0.20**	**0.02‡**	1	-	0.07	0.45	**0.20**	**0.03‡**
Human OSW*	0.07	0.42	1	-	**0.18**	**0.04‡**	0.07	0.45	1	-	**0.18**	**0.05‡**
Headache†	0.06	0.53	0.03	0.77	-0.04	0.70	0.10	0.32	0.02	0.83	-0.02	0.87
Muscle†	-0.14	0.11	0.13	0.15	-0.08	0.40	-0.06	0.53	0.14	0.15	-0.05	0.64
Fever†	0.06	0.52	0.06	0.49	0.06	0.55	0.11	0.24	0.06	0.53	0.04	0.65
Runny nose†	-0.01	0.91	0.18	0.49	0.06	0.50	0.02	0.87	0.01	0.89	-0.01	0.95
Sore throat†	0.04	0.64	0.02	0.79	-0.02	0.80	**0.18**	**0.05‡**	0.01	0.89	-0.01	0.95
Sputum†	0.17	0.06	0.06	0.54	0.06	0.49	0.18	0.06	0.05	0.62	0.08	0.39
Cough†	0.09	0.33	0.07	0.43	0.06	0.51	**0.21**	**0.03‡**	0.07	0.46	0.09	0.36
Pig pool average*	**0.20**	**0.02‡**	**0.18**	**0.04‡**	1	-	**0.20**	**0.03‡**	**0.18**	**0.05‡**	1	-

* Pearson Correlation test
† Spearman Correlation test
‡ Significant correlation at the level of P=0.05 (bilateral).



Green: Negative sample; Red: Positive Sample; Orange: Suspect Sample; White: No sample.

Figure 4.2 Heat map showing the comparison between two cut-offs of Matrix RT-qPCR results from human and swine samples per month. A month result was defined as positive (red) if one sample per farm per month was positive; and as negative (green) if no positive samples were found during the indicated month in a particular farm.

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APPENDIX

Table S3.1 Characteristics of the humans enrolled in the study (N = 26).

Characteristic	N	%
Site (Localization)		
Alberta	2	7.7
Saskatchewan	10	38.5
Manitoba	14	53.8
Total	26	100
Gender		
Male	18	69.2
Female	6	23.1
No Answer	2	7.7
Total	26	100
Age Range		
18-25 Years	1	3.8
25-35 Years	8	30.8
35-45 Years	8	30.8
45-55 Years	7	26.9
No Answer	2	7.7
Total	26	100
Operation Site		
Farrowing	10	38.5
Nursery	3	11.5
Grower-Finisher	4	15.4
Breeding-Gestation	9	34.6
All Sites	4	15.4
Q Barn	1	3.8
Maintenance	1	3.8
Office	1	3.8
No Answer	2	7.7
Total	71	134.5

Note. Total of percentages in the Operation site is not 100 because participants sometimes worked in more of one area.

Table S3.2 Summary of participation of human subjects per month from October 2015 to April 2016.

Month	Number of Participants (n)	Percentage (%)
October	7	26.9
November	18	69.2
December	19	73.1
January	21	80.8
February	22	84.6
March	21	80.8
April	22	84.6

Percentage calculated with the total number of participants enrolled in the study (N=26).

Table S3.3 Symptom score frequency of all the symptoms evaluated.

Symptom/ Score	Headache (N)	%	Muscle pain (N)	%	Fever (N)	%	Runny nose (N)	%	Scratchy Throat (N)	%	Sputum	%	Cough (N)	%
Never	50	39.7	26	20.6	84	67.2	46	36.5	52	40.9	50	40.3	40	32.5
Occas.	62	49.2	70	55.6	35	28	59	46.8	59	46.5	42	33.9	60	48.8
Often	11	8.7	18	14.3	2	1.6	15	11.9	9	7.1	22	17.7	16	13
Very Often	3	2.4	12	9.5	4	3.2	6	4.8	7	7	10	8.1	7	5.7

Note: The total N was based on the responses received. Occas. = Occasional

Table S3.4 Results of the Real-time RT-PCR of human nasal swabs and oral swabs.

Results	Nasal Swabs (n)	Percentage (%)	Oral Swabs (n)	Percentage (%)
Negative	129	99.23	127	97.69
Positive	1	0.77	3	2.31
Total	130	100	130	100

Note: The percentage for the negative, positive and suspect variables is based on the total number of received samples.

Table S3.5 Subtypes isolated from human nasal and oral swabs.

Subtype	Nasal Swab (n)	Percentage (%)	Oral Swab (n)	Percentage (%)
No subtype	1	100	3	33.33
H1	0	0	2	66.67

Table S3.6 Pearson Correlation results from swine Matrix PCR results by pool and comparison with a Ct average value for the three pools.

	Pig Pool 1		**Pig Pool 2**		**Pig Pool 3**		**Pig Pool Average**	
Variable	α	P	α	P	α	P	α	P
Pig Pool 1	-	-	0.789	0.000ß	0.679	0.000ß	0.923	0.000ß
Pig Pool 2	0.789	0.000ß	-	-	0.638	0.000ß	0.896	0.000ß
Pig Pool 3	0.679	0.000ß	0.638	0.000ß	-	-	0.866	0.000ß
Pig Pool Average	0.923	0.000ß	0.896	0.000ß	0.896	0.000ß	-	-

ß Significant correlation at the level of 0.01 (bilateral).

Table S3.7 IAV-S subtypes from swine nasal swabs from all the farm during the study.

Subtype	N	Percentage (%)
H1N1	1	2.94
H1N2	27	79.41
H3	5	14.71
H1	1	2.94

Note: Total N corresponds to the total number of pools that could be subtyped (N=34)