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2017

Novel insights in the adaptation of avian H9N2 influenza viruses to swine

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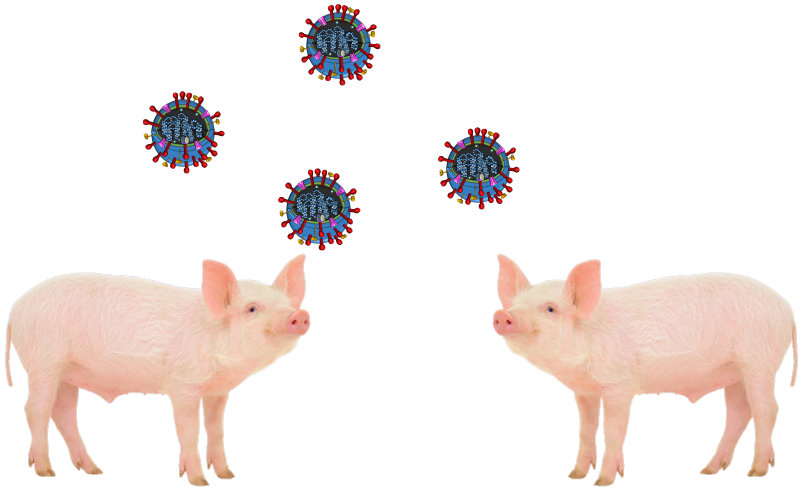

José Carlos Mancera Gracia

2017

Novel insights in the adaptation of avian H9N2 influenza viruses to swine

José Carlos Mancera Gracia

Doctor of Philosophy (Ph.D.) in Veterinary Sciences

2017

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“I am fond of pigs. Dogs look up to us. Cats look down to us. Pigs treat us as equals.”

Winston Churchill
United Kingdom Prime Minister 1940-1945
and 1951-1955

A mis padres, Inmaculada y José Carlos.

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LIST OF ABBREVIATIONS

A	aa	amino acid
	AEC	3-amino-9-ethyl-carbazole
	AIV	avian influenza virus
	AUC	area under the curve
B	bp	base pairs
	BSL	biosafety level
C	CDC	Centers for Disease Control and Prevention
	cDNA	complementary deoxyribonucleic acid
	CI	confidence interval
	cRNA	complementary ribonucleic acid
D	dpc	days post-contact
	dpi	days post-inoculation
E	ELISA	enzyme-linked immunosorbent assay
F	FAO	Food and Agriculture Organization
G	GOF	gain-of-function
H	H1N2v	H1N2 variant
	H3N2v	H3N2 variant
	HA	hemagglutinin
	HEPA	high efficiency particle arresting
	HI	hemagglutination inhibition
	HPAIV	highly pathogenic avian influenza viruses
L	LPAIV	low pathogenic avian influenza viruses
M	M	matrix protein
	M1	matrix protein 1
	M2	matrix protein 2

	MDCK	Madin-Darby canine kidney
	MOI	multiplicity of infection
	mRNA	messenger ribonucleic acid
N	NA	neuraminidase
	NEP	nuclear export protein
	Neu5Ac	non-O-acetylated N-acetylneuraminic acid
	Neu5Gc	N-glycolylneuraminic acid
	ng	nanogram
	NGS	next generation sequencing
	nM	nanoMolar
	NP	nucleocapsid protein
	NS1	non-structural protein 1
	NS2	non-structural protein 2
O	OIE	World Organization for Animal Health
	OFFLU	OIE-FAO global network of expertise on animal influenza
P	PA	polymerase acidic
	PB1	polymerase basic 1
	PB2	polymerase basic 2
	PBS	phosphate-buffered saline
	pH1N1	2009 pandemic H1N1
	PRDC	porcine respiratory disease complex
	PRRS	porcine reproductive and respiratory syndrome
R	R_0	basic reproduction ratio
	RBS	receptor-binding site
	RNA	ribonucleic acid
	RNP	ribonucleoprotein
	RT-PCR	reverse transcription-polymerase chain reaction

S	Sia	sialic acid
	Siaα2,3Gal	α2,3Gal-linked sialic acids
	Siaα2,6Gal	α2,6Gal-linked sialic acids
	SIV	swine influenza virus
T	$TCID_{50}$	tissue culture infectious dose with a 50% end point
	TRIG	triple-reassortant internal genes
U	URT	upper respiratory tract
	US	United States
V	vRNA	viral ribonucleic acid
W	WHO	World Health Organization

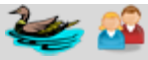
1 GENERAL INTRODUCTION

1.1 Introduction to influenza viruses

1.1.1 Taxonomy and virion structure

Influenza viruses cause a highly contagious respiratory disease known as influenza or "flu", which has posed a significant threat for animals and humans since the ancient times. These viruses are the most important members of the family *Orthomyxoviridae*. Their genome is composed of 7 to 8 segments of negative-sense single-stranded ribonucleic acid (RNA). Influenza viruses are divided into 4 genera/types: A, B, C and D. This classification (into the 4 genera) is based on the genetic and antigenic differences between their nucleocapsid (NP) and their matrix 1 (M1) proteins (Wright et al., 2013). Influenza B and C viruses are largely restricted to humans but have also been sporadically isolated from other mammals (Kimura et al., 1997, Hause et al., 2013). Since 2011 influenza D viruses have been identified in cattle and pigs (Ducatez et al., 2015, Chiapponi et al., 2016, Ferguson et al., 2016). Influenza B, C and D viruses are considered a lower threat to public and veterinary health, and they will not be further discussed in this thesis. In contrast, influenza A viruses are of great importance because they can infect a wide variety of avian and mammalian species (Fouchier et al., 2003, Harder and Vahlenkamp, 2010). These viruses are further divided into subtypes based on the antigenic properties of their surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). As of today 18 different HAs (H1-H18) and 11 NAs (N1-N11) have been identified (Fouchier et al., 2005, Mehle, 2014). With the exception of subtypes H17N10 and H18N11, which were only found in bats, all known subtypes of influenza viruses are established in birds. Yet just a limited number of subtypes have become endemic in humans and pigs (Table 1).

Table 1. Overview of HA and NA subtypes of influenza A viruses that are or have been endemic in birds, humans, pigs and other animal species (adapted from the US CDC: http://www.cdc.gov/flu/about/viruses/transmission.htm).

HA		NA	
Subtype	**Host species**	**Subtype**	**Host species**
H1		N1	
H2		N2	
H3		N3	
H4		N4	
H5		N5	
H6		N6	
H7		N7	
H8		N8	
H9		N9	
H10		N10	
H11		N11	
H12			
H13			
H14			
H15			
H16			
H17			
H18			

The current nomenclature system for influenza viruses gives information about the type, host, place of isolation, strain number, year of isolation and antigenic subtype of the virus. For instance, an H9N2 virus isolated from quail in Hong Kong in 1997 was designated as A/quail/Hong Kong/G1/1997 (H9N2).

Influenza A virions are spherical or pleomorphic with a diameter between 80 and 120 nanometers. They are enveloped viruses and encode 10 viral proteins on 8 separate segments of negative-sense RNA. A few additional proteins were only recently identified (Shi et al., 2014). The envelope is a lipid bilayer derived from the host cell membrane and contains the HA and the NA that play a crucial role in the virus binding and release, respectively (Figure 1). Another transmembrane protein, the matrix protein 2 (M2), is a type III proton selective ion-channel essential in the release of the uncoated ribonucleoproteins (RNPs) into the cytoplasm of the host cell. Just underneath the virion envelope, the M1 forms a layer that interacts with both the envelope and the RNPs. This M1 is responsible for the structural integrity of the virion and encapsidates the 8 RNPs. The RNPs consist of a RNA segment, NPs and the polymerase complex (PA, PB1 and PB2) that is responsible for viral RNA (vRNA) replication and transcription. The non-structural (NS) segment encodes 2 proteins: NS1, a host antiviral response antagonist and NS2 (also named nuclear export protein NEP), which is associated with the M1 and mediates the export of the RNPs from the nucleus to the cytoplasm.

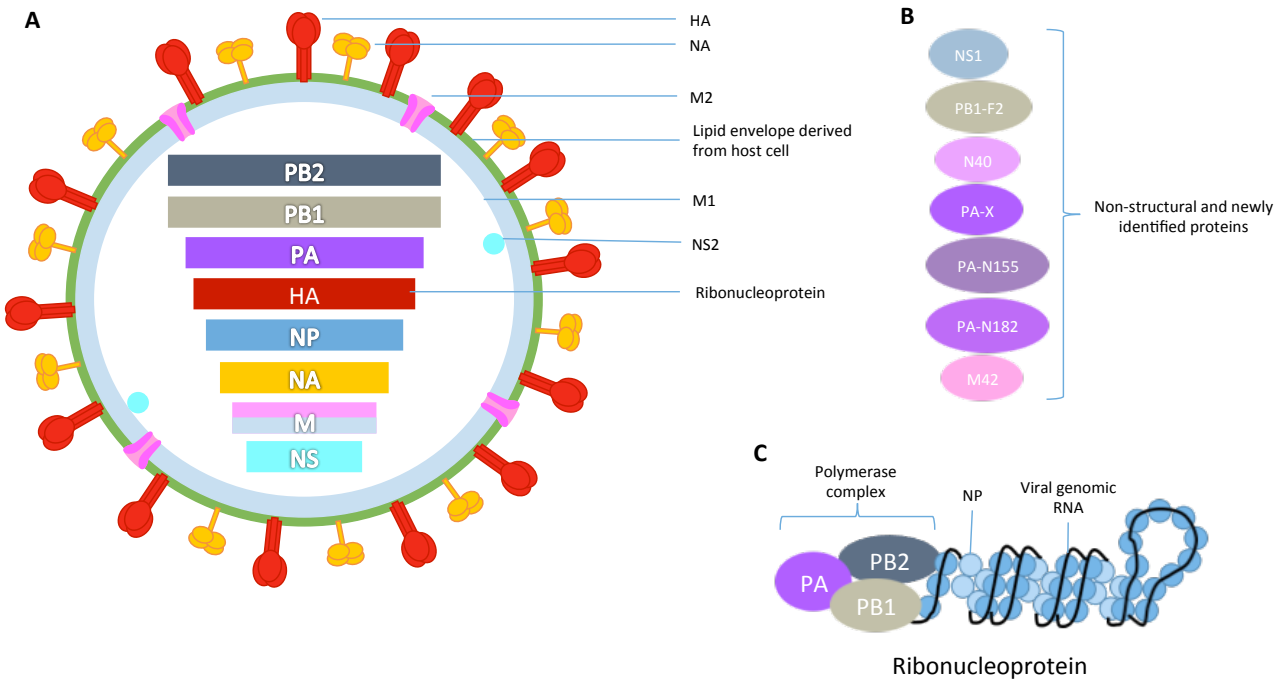


Figure 1. (**A**) The structure of influenza A virion. (**B**) Non-structural proteins identified, the colour of each protein matches the colour of the gene-segment that codes it. (**C**) Representation of the influenza A ribonucleoprotein (adapted from Shi et al. 2014).

1.1.2 Replication cycle

The infection of a susceptible host cell is initiated by the interaction of the HA receptor-binding site (RBS) with terminal sialic acids (Sia) linked to glycoproteins or glycolipids on the host cell membrane (see Figure 2). The contact between Sia and the HA is established by low affinity bonds such as hydrogen bonds, hydrophobic interactions and van der Waals contacts (Gamblin and Skehel, 2010). Therefore, binding of multiple cell receptors to multiple HA spikes is needed to obtain a high binding avidity. Upon binding to the host cell Sia residues, a clathrin-mediated or clathrin- and caveolin-independent endocytosis occurs and, as a result the virus enters in the host cell in an endosome (Skehel and Wiley, 2000, Sieczkarski and Whittaker, 2002, Lakadamyali et al., 2004). During the migration from the cell surface towards the nucleus, the viral and the endosomal membranes fuse. This fusion is triggered by conformational changes produced in the HA by a low pH environment (Cross et al., 2009). Moreover, the low pH present in the endosome

opens the M2 ion-channel creating an additional influx of protons into the virus interior, which causes the disconnection of the RNPs from the M1 proteins (Pinto and Lamb, 2006). The RNPs are subsequently released into the cytoplasm, and migrate to the nucleus, where transcription and replication occur. So as to enter into the nucleus, the RNPs use known nuclear localization signals that bind to the cellular nuclear import machinery (Boulo et al., 2007). As influenza A viruses possess negative-stranded RNA, a complementary copy of it must be made to obtain the viral messenger RNA (mRNA). Furthermore, vRNA does not contain 5´methylated caps that are needed to complete the synthesis of mRNA (Das et al., 2010). Thus, to overcome that situation, the 5'caps of cellular host derived pre-mRNA are bound by the PB2 and cleaved-off by the endonuclease activity of PA at approximately 10 to 13 nucleotides from the cap structure in a process called "cap-snatching" (Guilligay et al., 2008, Dias et al., 2009). This cellular capped RNA fragment is subsequently used for priming viral transcription by the viral polymerases (Reich et al., 2014). The production of new vRNA requires the synthesis of an intermediate positive-sense template, called complementary RNA (cRNA), which serves as template for the production of progeny RNA segments in the nucleus. All proteins comprising the RNP migrate from the site of synthesis in the cytoplasm to the nucleus where they merge with vRNA. The interaction of M1, NS2 and cellular nucleoporins prompts the nuclear RNP export into the inner surface of the plasma membrane (Neumann et al., 2000). There, the 8 gene segments are gathered together by packaging the RNP segments, NS2 and M1 underneath the apical cell membrane. Virus assembly occurs while HA, NA and M2 are already embedded in the apical plasma membrane. The budding of the virus also involves M1 proteins and several host factors (Nayak et al., 2009). The last step constitutes the release of the newly formed virion. During this process NA acts as a "razor" that enzymatically shaves the Sia residues from the glycoproteins and glycolipids present on the cell surface allowing an efficient virus release (Gamblin and Skehel, 2010).

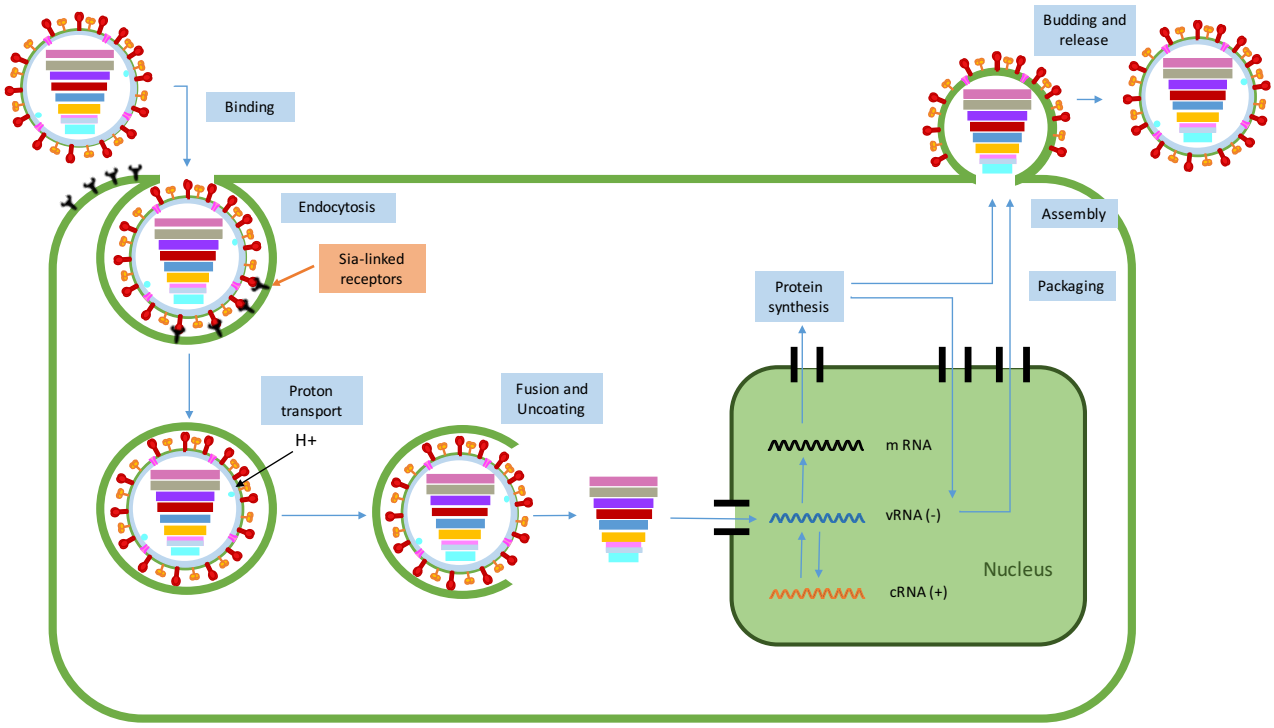


Figure 2. Schematic overview of the influenza A virus replication cycle (adapted from Shi et al., 2014).

1.1.3 Mechanisms of evolution and emergence of novel influenza viruses

Because of the nature of their viral genome, influenza A viruses carry their own polymerase genes, which lack exonuclease-proofreading capability. Therefore, influenza A viruses are evolutionarily dynamic and exist as populations of *quasispecies* (Domingo et al., 1998) with mutation rates ranging from 1 x 10^{-3} to 8 x 10^{-3} substitutions per site per year (Chen and Holmes, 2006). The surface glycoproteins, HA and NA, are the most important antigens for inducing protective immunity in the host, but also the most variable genes. Mutations that change amino acids in the antigenic sites of those proteins may allow influenza viruses to escape from pre-existing immunity. Such selective mutations produced in the HA and NA antigenic domains are known as “antigenic drift”. It can produce selective advantages for viral strains, and it is the reason why the virus strains present in the human influenza vaccines need to be reviewed every year.

Due to the presence of 8 independent RNA segments in the influenza A virus genome, simultaneous co-infection of a host cell with 2 or more different viruses can result in progeny viruses containing a novel combination of gene segments from

both parental viruses. This phenomenon is known as genetic “reassortment”. When the genetic reassortment results in the emergence of a virus containing novel HA and/or NA proteins it is designated as “antigenic shift” (Taubenberger and Kash, 2010). The combination of antigenic drift and shift allows influenza viruses to constantly reinvent themselves posing a continuous threat to animal and human health (Li and Chen, 2014).

1.2 Ecology and pathogenesis of avian, human and swine influenza viruses

1.2.1 Avian influenza viruses

Wild aquatic birds (orders *Anseriformes* and *Charadriiformes*), especially ducks, shorebirds and gulls are natural hosts for most influenza A virus subtypes (Sorrell et al., 2007). As much as 82 different HA and NA combinations have been isolated from aquatic birds (Olsen et al., 2006). Avian influenza viruses (AIVs) primarily replicate in the cells of the intestinal epithelium of birds, and are excreted in high concentrations in their faeces. As a consequence, wild birds transmit AIVs via the faecal-oral route. Thus migrating aquatic birds can carry viruses between their summer and winter habitats, spreading the viruses during their feeding stops (Munster et al., 2006). Influenza A virus infections in wild aquatic birds are generally asymptomatic (Olsen et al., 2006).

Land-based poultry such as chickens, turkeys, quails and pheasants are also susceptible to influenza A viruses (Alexander, 2000). In these species, influenza A viruses are classified into the following 2 categories based on molecular characteristics and the ability of the virus to cause disease and mortality in chickens in a laboratory setting:

Low pathogenic avian influenza viruses (LPAIV):

Most AIVs are LPAIV. Infection of poultry with this pathotype usually causes a subclinical or mild disease with symptoms such as ruffled feathers and/or a drop in egg production. However, in chickens and turkeys LPAIV infection may also result in respiratory disease, sinusitis and depression, which can be aggravated by secondary bacterial infections. The most frequently isolated subtypes from poultry are: H1, H3, H5, H6, H7 and H9 (Alexander, 2000, Perez and de Wit, 2017 and Sims et al., 2017).

Highly pathogenic avian influenza viruses (HPAIV):

AIVs of H5 and H7 subtypes may become HPAIV upon introduction from wild aquatic birds into poultry. All HPAIVs have arisen by the insertion of multiple basic amino acid (aa) residues in the HA cleavage site of LPAIVs. This cleavage site is known to process the HA precursor (HA0) to the functional components HA1 and HA2 (Alexander and Brown, 2009). A basic cleavage site allows cleavage of HA by widely distributed cellular proteases that do not cleave LPAIV HAs. This facilitates replication in a wider range of organs resulting in a more severe illness with morbidity and mortality rates up to 100%. HPAIVs have been mainly isolated from chickens and turkeys. The typical signs in those species include decreased egg production, respiratory signs, lacrimation, sinusitis, cyanosis of combs and wattles, oedema of the head, ruffled feathers, diarrhoea and nervous symptoms. However, these signs are variable depending on the host species and age, the virus strain, and the possible concurrence of secondary bacterial infections (Webster et al., 1992, Sims et al. 2017). The mechanisms leading to the switch from LPAIV to HPAIV viruses in poultry are not completely understood. Therefore, it is impossible to predict when HPAIV will emerge. Nevertheless, it is assumed that the odds increase in direct proportion with the circulation of H5 and H7 LPAIVs within poultry flocks.

1.2.2 Human influenza viruses

Influenza A viruses circulate in humans as an annually recurrent epidemic disease that causes millions of human infections worldwide and has significant economic and health burdens (Medina and Garcia-Sastre, 2011). Only 3 specific subtypes (H1N1, H2N2 and H3N2) have reached the endemic status in humans since 1918 (Figure 3) (Taubenberger and Kash, 2010). The high population immunity forces human influenza viruses to change continuously. Changes occur mainly in the viral HA and NA and may lead to epidemics and pandemics.

The antigenic evolution of circulating strains could result in an “antigenic drift” that causes an epidemic outbreak affecting more than the expected number of people in a community or region during a given period of time. When an epidemic virus spread in human population across a whole continent or even the world it generates a pandemic. The generation of a novel pandemic virus depends on 2 main factors: the introduction of an antigenically novel HA subtype in the human population, and the ability of the virus to spread efficiently between humans. Even though the exact

mechanisms by which pandemic influenza A viruses are generated are still poorly understood, 3 different mechanisms have been suggested. A first mechanism is the introduction in humans of a novel virus subtype from a different host. The 1918 pandemic H1N1 virus, also known as “Spanish flu”, is believed to result from such an event. This H1N1 pandemic virus caused ≈ 50 million deaths worldwide. Recently all genes of this virus were shown to be avian-like (Taubenberger and Morens, 2006). The second mechanism is the generation of a virus containing a novel HA and/or NA subtype by genetic reassortment between animal and human influenza viruses. The 1957 H2N2 pandemic, also known as “Asian flu”, was generated by reassortment between an avian virus that donated the HA, NA and PB1 gene-segments, and the then circulating human H1N1 strain. Another example is the 1968 H3N2 “Hong Kong flu” pandemic. This virus acquired avian-origin HA and PB1 gene-segments and the remaining 6 segments from the human H2N2 virus that was circulating in 1968 (Taubenberger and Kash, 2010). The third mechanism is the re-introduction of a HA that disappeared from the human population but that has been kept in stasis in another reservoir species. Through this mechanism an earlier circulating strain may be re-introduced into humans from a non-human reservoir when the human population immunity has become very low. The H1N1 virus that generated the 1977 pandemic, named the “Russian flu”, was most likely the result of a re-introduction of the human H1N1 virus that has been circulating from 1918 up until the emergence of H2N2 pandemic in 1957 (Taubenberger and Kash, 2010). Genetic analyses support the belief that the reintroduction was due to the accidental release of a 1950s frozen virus from a laboratory (Nakajima et al., 1978). A better example of the third mechanism is the 2009 pandemic H1N1 virus (pH1N1). Interestingly, this virus belongs to H1 subtype, which has been circulating in humans for decades. However, pH1N1 HA protein is antigenically very distinct from the human-seasonal H1N1 viruses that were circulating in 2008-09. It contains a “classical swine” H1 HA, which is derived from the 1918 pandemic H1N1 influenza viruses and that remained in antigenic stasis in swine (see Figure 4). Consequently, people born after 1950s were immunologically naïve to pH1N1 (Kash et al., 2010).

In humans, seasonal influenza infections are restricted to the respiratory tract and are characterized by a high morbidity and low mortality. Figure 3 depicts the evolution of the pandemic influenza viruses reported since 1918.

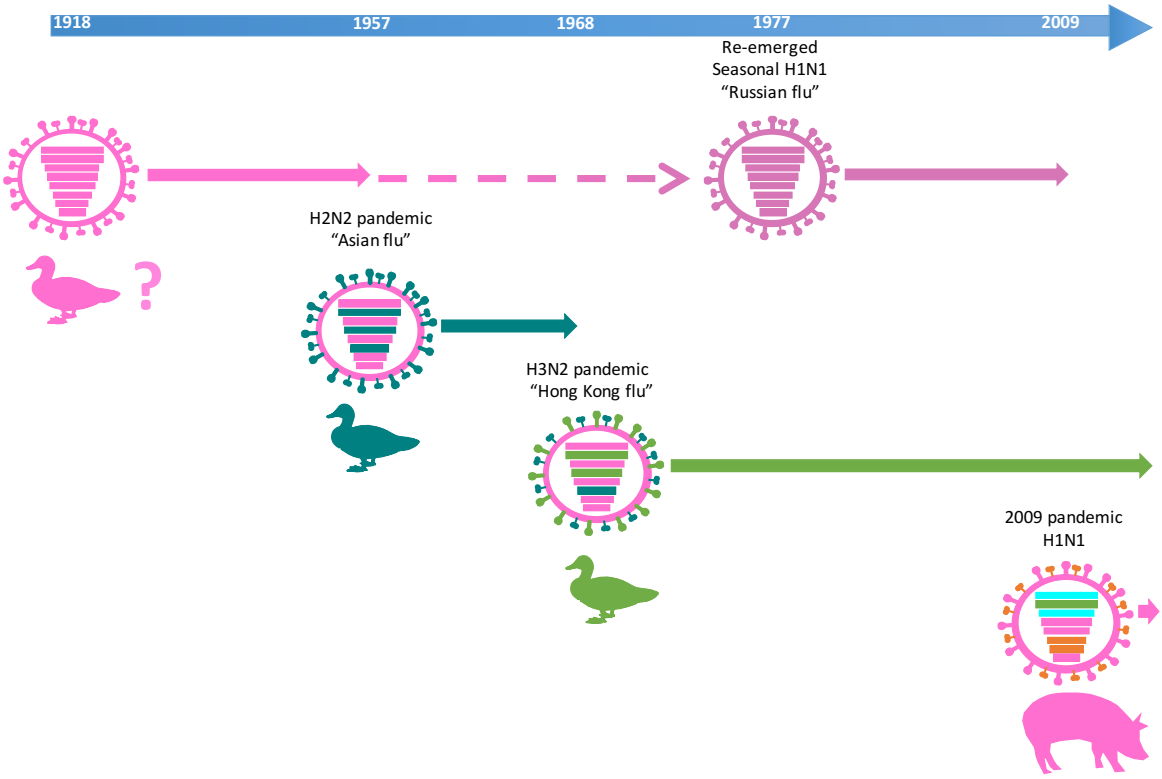


Figure 3. Origin of the 4 known pandemic influenza A viruses in humans since 1918.

1.2.3 Swine influenza viruses

In pigs, influenza A viruses are one of the major causes of acute respiratory disease outbreaks. Although "influenza A virus in swine" is the nomenclature recommended by the OIE-FAO Global Network of Expertise on Animal Influenza (OFFLU) for influenza A viruses that are endemic in pigs, in this thesis we will use the traditional designation "swine influenza virus (SIV)" (OIE, 2017). SIVs may contribute to the so-called porcine respiratory disease complex (PRDC) in concert with other agents such as porcine reproductive and respiratory syndrome (PRRS) virus and *Mycoplasma hyopneumniae*, generating multifactorial disease problems. Though only 3 influenza virus subtypes (H1N1, H3N2 and H1N2) are currently circulating in swine worldwide, the origins and the antigenic characteristics of these SIV subtypes are different in different regions of the world (Figure 4). In Europe, the first significant SIV outbreaks

occurred in 1979 after the transmission of an avian H1N1 virus from wild ducks to pigs in Belgium and Germany (Pensaert et al., 1981). This European "avian-like" H1N1 virus rapidly spread throughout Europe, and nowadays it is still the dominant subtype in many European regions (Simon et al., 2014). Later, in the 1980s, reassortant viruses containing human-seasonal H3N2 surface protein genes in the European avian-like H1N1 backbone emerged and became endemic in European pigs (Simon et al., 2014). Since the mid-1990s, those European H3N2 SIVs reassorted with a human-seasonal H1N1 virus HA generating the H1N2 virus lineage that became established throughout Europe (Brown et al., 1998, Van Reeth et al., 2000). For many years, those 3 SIV lineages have been co-circulating in the European swine population keeping the epidemiological situation rather stable (Simon et al., 2014, Watson et al., 2015). However, this situation changed with emergence of the pH1N1 virus (Watson et al., 2015). This virus has become endemic in European pigs complicating the swine influenza epidemiology in many cases due to its reassortment with pre-existing H1N1, H3N2 and H1N2 SIVs (Harder et al., 2013, Lange et al., 2013, Moreno et al., 2013, Rose et al., 2013, Simon et al., 2014, Lewis et al., 2016).

In North America the epidemiological situation was stable for nearly 80 years. The "classical swine" H1N1 was the dominant subtype in the US since its first observation in 1918. However, this situation dramatically changed in 1998, when "triple-reassortant" H3N2 viruses containing gene segments from the "classical swine" H1N1 viruses (NP, M, NS), human-seasonal H3N2 viruses (PB1, HA and NA) and AIVs (PB2 and PA) became successfully established in the pig populations (Zhou et al., 1999, Webby et al., 2000). These "triple-reassortant" H3N2 viruses co-circulated with "classical swine" H1N1 viruses and subsequent reassortments led to the generation and spread of "triple-reassortant" H1N1 or H1N2 viruses (Karasin et al., 2002, Webby et al., 2004, Vincent et al., 2008). In addition, these H1 "triple-reassortant" viruses also reassorted with human-seasonal H1 viruses generating H1N1 and H1N2 reassortants containing human-origin H1 HA (Lewis et al., 2016). As seen in Europe, the introduction of the pH1N1 virus and its reassortment with endemic North American SIVs has also deeply changed swine influenza epidemiology (Kitikoon et al., 2013, Rajao et al., 2016). The pH1N1 is a reassortant virus originated

in Mexican swine by the combination of 2 different swine influenza viruses (SIVs) circulating in the North America and Europe, see figure 4 (Smith et al., 2009, Mena et al., 2016). Indeed, novel H3N2, H1N1 and H1N2 viruses containing pH1N1 internal genes have spread in the US swine population and have been recurrently isolated from humans since 2011, raising public health concerns. These viruses with the ability to infect humans were denominated as "variant" viruses (Kitikoon et al., 2013, Centers for Disease Control and Prevention, 2016).

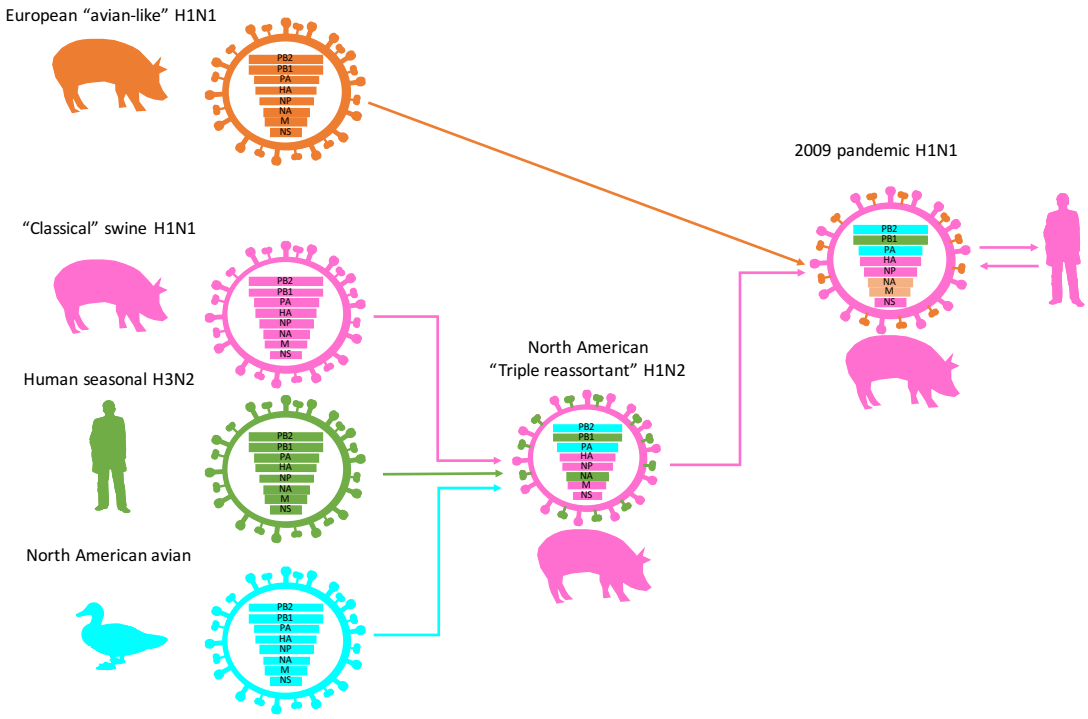


Figure 4. Origin of the 2009 pandemic H1N1 virus. The NA and M protein-genes derived from the European "avian-like" H1N1 virus and the remaining protein-genes derived from those of the North American "Triple reassortant" H1N2 virus (adapted from Neumann et al., 2009).

In South America and Asia the epidemiology of SIVs is different when compared to Europe and North America (Pereda et al., 2010, Cappuccio et al., 2011,). For instance, in Asia viruses from Europe and North America have been introduced and co-circulate with local strains (Yu et al., 2009, Vijaykrishna et al., 2010, Ngo et al., 2012). In consequence, the co-circulation of so many genetically diverse SIVs has led to a very complex collection of reassortant viruses.

1.3 Interspecies transmission and the species barrier

1.3.1 Transmission from birds to mammals

Influenza A viruses infections are generally species-specific. There is a strong species barrier that restricts the transmission to novel hosts. Actually, wild aquatic birds are considered a reservoir for influenza A viruses in mammals. However, AIVs have to overcome that strong species barrier to become efficiently adapted to mammals. The likelihood of these viruses to successfully overcome that barrier depends on 2 main types of interactions.

Host-host interactions:

Infections of mammals with AIVs are in most of the cases dead-end events. However, if the interactions between the donor and the recipient hosts become common it will increase the likelihood of the viruses to overcome the species barrier. For example, the live-poultry markets (wet-markets) where chickens, ducks, geese, quails and other species of birds are sold for human consumption have been suggested as a risk for the introduction of AIVs into mammals (Offeddu et al., 2016). Then, assuming that the virus can be transmitted between individuals of the new host species, infections will depend on interactions between recipient hosts.

Virus-host interactions:

Virus-host interactions are the steps that influenza viruses have to accomplish to efficiently infect a new host. First, these viruses have to deal with the mucins present in the respiratory tract secretions. Those mucins can specifically bind them interfering their binding to the airway epithelial cells (Yang et al., 2014). The following interaction is the successful attachment to the receptors present on the host cell surface. Once they have entered the cell the viruses must take over host cell processes to replicate there. Finally, they need to be efficiently released from the cell in order to be able to infect new cells and to spread (Kuiken et al., 2014).

The mechanisms for adaptation of AIVs to mammals are still largely unknown. To increase the insight in influenza virus transmission 2 contrary experimental approaches are mainly used. The “loss-of-function” approach consists of modifications to viruses which have already been adapted to a novel host species. When they do not longer transmit between different subjects, the proteins or aa motifs that are essential for transmission can be identified. In contrast, “gain-of-

function" (GOF) experiments aim to achieve transmission with a non-adapted virus to better understand the mechanisms for adaptation (Imai et al., 2013). Whereas the first approach may incur a lower biosafety risk, the second gives more insights in the exact mechanisms that rule the adaptation.

1.3.2 Transmission between humans and swine

Zoonotic infections with SIVs have been reported worldwide since 1958. In 1976, the first large-scale case of transmission of SIV among humans occurred on a military base in Fort Dix, New Jersey. Up to 230 soldiers were diagnosed with "classical" H1N1 SIV infection using serological investigation (Gaydos et al., 2006). Later on, swine-to-human transmission events were sporadic. Until 2009, with the exception of the SIV outbreak which had occurred at Fort Dix, there was limited evidence for person-to-person spread of swine-origin viruses (Krueger and Gray, 2013). Then the 2009 pH1N1 virus appeared and spread worldwide, being the first pandemic influenza virus of proven swine origin (Mena et al., 2016). Furthermore, the pH1N1 reassorted with other endemic SIVs generating viruses with the ability to infect humans such as the H3N2v that has been isolated from 402 humans in the US since 2011 (World Health Organization, 2017a). The emergence of the pH1N1 virus dramatically changed the understanding of SIV ecology and cross-species transmission because it was efficiently transmitted from swine-to-humans, but also from humans-to-swine.

A recent study demonstrated that transmission from humans-to-swine is far more frequent than the other way around (Nelson and Vincent, 2015). Actually, reassortant viruses containing human-origin gene segments have become widespread in swine worldwide (Watson et al., 2015, Lewis et al., 2016). For instance, the European H3N2 SIVs were generated upon the spread and reassortment of a human-seasonal H3N2 "Port Chalmers/75-like" virus (Brown, 2000). However, the lack of surveillance makes it difficult to accurately estimate the extent of transmission of human-seasonal viruses to swine in the field. Experimental studies also confirm the susceptibility of pigs to human viruses. In fact, the inoculation of pigs with a human H3N2 or a reconstituted 1918 pandemic H1N1 virus resulted in transient fever, mild respiratory signs and nasal virus excretion. However, those viruses were not able to achieve efficient pig-to-pig transmission (Landolt et

al., 2006, Weingartl et al., 2009). Although it has been demonstrated that transmission of influenza A viruses between pigs and humans occurs, until now only the pH1N1 has been demonstrated to be able to efficiently transmit and spread in both species.

1.3.3 Avian influenza viruses with pandemic potential

Between 1959 and 1996 only 3 cases of AIVs infecting humans have been reported. Consequently, AIVs were considered as a low risk for human health. However, since the first isolation of an H5N1 HPAIV in Hong Kong in 1997, AIVs became a cause of concern. Furthermore, the number of zoonotic AIV infections has dramatically increased during the last decade. This section will highlight the avian influenza subtypes with the highest pandemic potential according to the World Health Organization (WHO) (World Health Organization, 2017 a).

H5 Subtype

So far, only influenza A H5N6 and H5N1 virus subtypes have been reported to infect humans (Yang et al. 2015, World Health Organization, 2017a). Since 2014, H5N6 HPAIVs have been sporadically isolated from humans in China, but the number of cases and their geographical spread remain very limited. In contrast, the H5N1 HPAIVs are on top of the list of avian influenza subtypes with the pandemic potential (World Health Organization, 2017a). As mentioned, the first human outbreak produced by H5N1 HPAIVs occurred in Hong Kong in 1997, causing severe disease with high fatality rates (around 60 %) (Claas et al., 1998, Subbarao et al., 1998). This H5N1 virus was indeed a reassortant virus containing a H5 HA derived from goose H5N1 virus and the remaining genes from H9N2 and H6N1 viruses prevalent in quail, as shown in Figure 5 (Guan et al., 1999, Hoffmann et al., 2000). Wet markets appeared to be the source of the human infection (Shortridge, 1999). Although the outbreak was aborted with the culling of 1.5 million poultry in farms and markets of Hong Kong, H5N1 HPAIV was again reported in humans in Hong Kong in 2003 (Peiris et al., 2004).

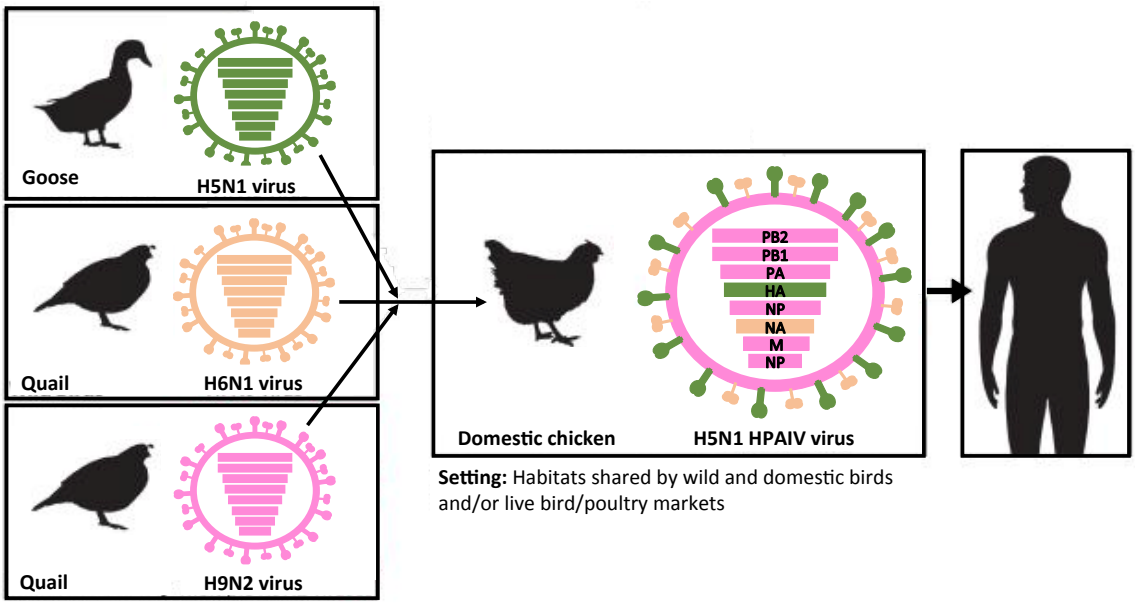


Figure 5. Origin and sources for the H5N1 HPAIV.

Later, human cases have also been reported in Vietnam, Thailand, Cambodia, Indonesia, Turkey, Iraq, Azerbaijan, Egypt, Djibouti and Nigeria (Peiris et al., 2007). As of May 2017 a total of 859 human cases have been reported worldwide, 453 (52.8%) of whom died (World Health Organization, 2017b). Most of those human cases were associated with direct contact with infected poultry and efficient transmission within humans was never reported. However, the evolution of H5N1 viruses is quick and its direction is still unclear. Significant genetic and antigenic evolution has occurred (Guan et al., 2004), involving antigenic drift in the HA, mutations in other genes, and reassortment with other AIVs (Chen et al., 2006, de Vries et al., 2015). Even though mammalian adaptation markers have been detected in some human and avian HPAIV H5N1 isolates, and human-to-human transmission between family members has been reported, there is no sustained human-to-human transmission (Ungchusak et al., 2005, Wang et al., 2008).

In order to unveil the requirements for transmission of H5N1 HPAIV in humans, 2 different GOF approaches were tested in ferrets, as they are considered the most important animal model for influenza transmission studies (Belser and Tumpey, 2017). In the first approach, Herfst et al. demonstrated efficient airborne transmission between ferrets of an avian H5N1 HPAIV containing only 5 aa substitutions. Those mutations were present in the HA and the PB2 gene-proteins, 3 out of 5 were artificially introduced in the virus and the remaining 2 emerged after

10 passages and 3 transmission experiments (all aa mutations mentioned in those adaptation experiments will be described in Chapter 1.4) (Herfst et al., 2012). The second approach evaluated if H5N1 viruses could become airborne transmissible in ferrets upon reassortment with contemporary human influenza viruses, with or without mammalian adaptation substitutions. On one hand, Imai et al. achieved efficient airborne transmission to 4 out of 6 contact ferrets after the introduction of 4 aa mutations in the HA of a reassortant virus containing H5 HA in the pH1N1 backbone (Imai et al., 2012). On the other hand, Chen et al. reached partial airborne transmission in ferrets by introducing key point mutations in the H5 HA RBS and a human-seasonal H3N2 NA gene (Chen et al. 2012). In summary, these studies demonstrate that H5N1 viruses can become adapted to ferrets in the laboratory either upon reassortment with human-adapted viruses (with the need for additional substitutions) or only by the introduction of certain key aa mutations.

H7 subtype

More than 100 human infections with H7N1, H7N2, H7N3 and H7N7 subtypes have been reported in Australia, Canada, Italy, Mexico, the Netherlands, the United Kingdom and the US. Most of these infections were associated with poultry outbreaks and resulted in conjunctivitis and mild upper respiratory symptoms with the exception of 1 death that occurred in the Netherlands (Fouchier et al., 2004, Stegeman et al., 2004, Nguyen-Van-Tam et al., 2006, Ostrowsky et al., 2012).

On March 2013, a novel influenza H7N9 LPAIV crossed the species barrier and caused for the first time human infections in China (Gao et al., 2013). Until May 2017, 1486 laboratory-confirmed human infections, including at least 571 deaths (40.1%), have been reported in China (World Health Organization, 2017a). This virus was likely transferred from domestic ducks to chickens before “jumping” to humans and it was the result of the reassortment of at least 4 origin viruses. This resulted in an H7N9 virus with a duck origin H7 HA, a duck origin (probably also wild bird) N9 NA and 2 different chicken origin H9N2 viruses internal genes, see Figure 6 (Lam et al., 2013, Liu et al., 2013).

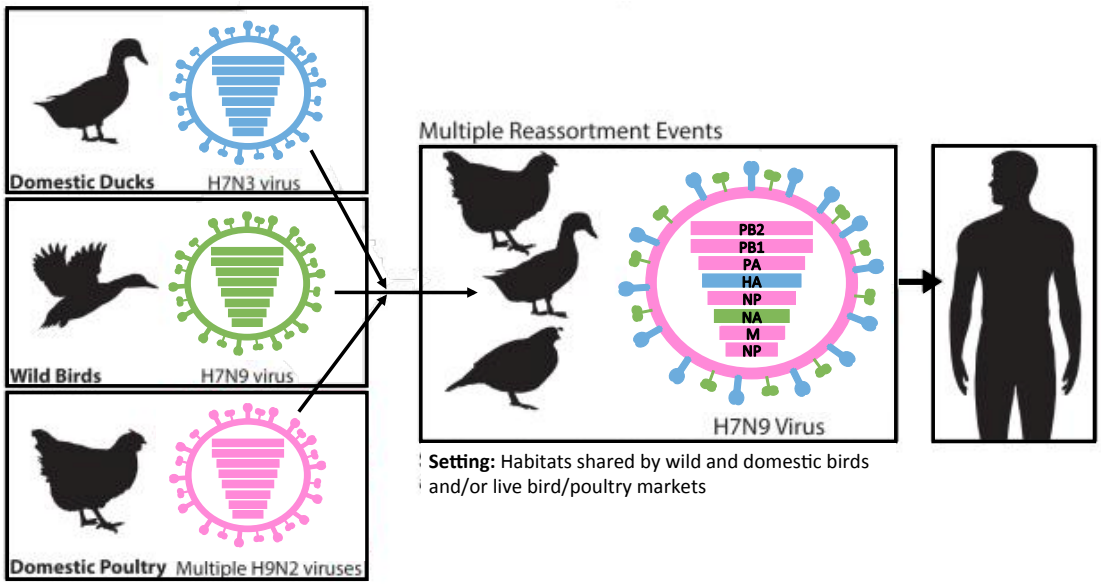


Figure 6. Origin and sources for the H7N9 influenza virus. (Adapted from Dan Higgins/PHIL CDC).

Epidemiological evidence has linked human cases with exposure to live-bird markets (Shi et al., 2013). The closure of these markets, with the likely assistance of environmental factors, helped to control the first wave of the outbreak from February to May 2013 (Yu et al., 2014). Nevertheless, public health efforts failed to completely stop the spread of human H7N9 infections and up to now 5 epidemic waves have been recorded. Although sporadic H7N9 virus transmission among family members has been reported, the likelihood of sustained human-to-human transmission is low (Qi et al., 2013, World Health Organization, 2017a).

Soon after its emergence in humans, several studies evaluated airborne transmission of avian-origin H7N9 viruses among ferrets (Richard et al., 2013, Belser et al., 2013, Zhu et al., 2013, Ku et al., 2014, Siegers et al., 2014). Interestingly, despite the fact that mutations associated with increased human adaptation were present in most human H7N9 virus isolates, efficient transmission in ferrets was never achieved (Luk et al., 2015, Neumann and Kawaoka, 2015). Hence, it is still unclear whether avian H7N9 viruses could become adapted to mammals. However, as long as those viruses continue circulating in poultry, human cases can be expected.

H9N2 subtype

H9N2 AIVs are endemic in wild waterfowl worldwide. This subtype has become endemic in different land-based poultry species across multiple Eurasian countries

(Guan et al., 1999, Sun et al., 2010, Sun and Liu, 2015, Van Borm et al., 2016). In 1999, H9N2 viruses were first isolated from humans in Hong Kong (Peiris et al., 1999). Since then, only 29 human cases have been reported and human-to-human transmission has never been described (World Health Organization, 2017a). These infections were associated with poultry outbreaks and mainly resulted in mild upper respiratory symptoms. Strikingly, a seroprevalence for H9N2 antibodies between 2.1% and 13.7% has recently been identified among poultry slaughterhouse workers in China (Huang et al., 2013). This finding suggests that H9N2 infections in humans go frequently unreported. As a matter of fact, most of the H9N2 AIVs circulating in poultry possess an aa substitution in the HA RBS that was described as a molecular marker for human adaptation, thereby increasing the possibility of these viruses to infect humans (Matrosovich et al., 2001, Wan and Perez, 2007). Interestingly, these viruses were also recurrently isolated from pigs in China since 1998.

As in H5 and H7 experimental studies, different approaches were also tested to evaluate potential of H9N2 viruses to become adapted to mammals. In 2008, Wan et al. evaluated virus replication and transmission in ferrets of different H9N2 wild-type viruses together with a reassortant containing H9N2 HA and NA in a human-seasonal H3N2 backbone. Although the reassortant virus replicated better than the wild-type viruses, efficient transmission was not reached (Wan et al., 2008). Later, upon ten passages of this reassortant in ferrets the virus acquired 2 aa mutations in the HA and 1 in the NA that were related with efficient airborne transmission (Sorrell et al., 2009). In a later experiment the same group demonstrated tested the compatibility of the previously used wild-type H9N2 with pH1N1 internal genes. Interestingly, this reassortant virus showed efficient airborne transmission in ferrets with no need of adaptive mutations. Moreover, this experiment also suggested that an optimal balance between HA and NA activity is required for efficient airborne transmission (Kimble et al., 2011). In addition, in 2013 different H9N2 strains were tested for replication and transmission in ducks, pigs, mice and ferrets in order to establish a ranking of H9N2 viruses that have the highest pandemic potential. At the end of the experiment, the human H9N2 virus isolates were on top of the list, but still those isolates were not as fit as the mammalian-adapted strains (The SJCEIRS H9 working group, 2013). In summary, efficient H9N2 adaptation to ferrets has been achieved by

reassortment with human-adapted viruses (with or without adaptive mutations). However, unlike in the H5N1 subtype, aa mutations that could confer efficient adaptation to those viruses are still unknown.

1.4 Molecular mechanisms of host range restriction

1.4.1 The role of the hemagglutinin

The main Sias recognized by the HA are the N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) (Matrosovich et al., 2015). Influenza A viruses can recognize the Sias bound to galactose in either an α2,3- or α2,6-glycosidic linkage (Connor et al., 1994, Matrosovich et al., 2009). These linkages are very important for the specificity of the HA molecules in the binding to cell surface sialic acids. While AIVs demonstrated highest preference for the α2,3Gal-linked Sias (Siaα2,3Gal), human and swine viruses preferentially bind α2,6Gal-linked Sias (Siaα2,6Gal) (Skehel and Wiley, 2000). The RBS is a shallow pocket placed in the HA1 that mediates binding to Sias present on host cell glycoproteins and glycolipids (Matrosovich et al., 2015). The aa residues making up this RBS showed to be very conserved and similar regardless of the virus subtype studied (Figure 7).

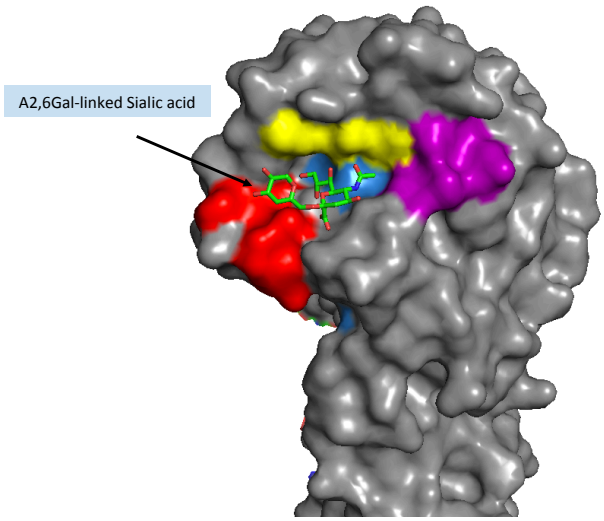
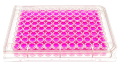
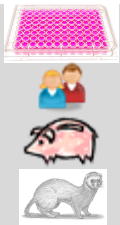
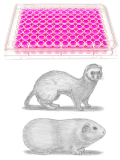
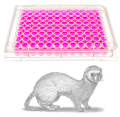


Figure 7. Surface representation of the RBS in the HA1 monomer of A/quail/Hong Kong/G1/1997 (H9N2) bearing an α2,6-linked sialic acid. The major structural components of the RBS are represented with the following colours: the central bottom in blue (amino acids (aa) 98, 153, 183 and 195), the rear in yellow (aa 155, 190 and 194), the right side in purple (aa 134 until 138) and the left side in red (aa 224 until 228) (Skehel and Wiley, 2000).

Therefore, aa changes in and around the RBS can dramatically alter the receptor binding preference of influenza viruses. Thus, a change in the receptor-binding preference of an influenza virus owing to mutations in the RBS is in most cases a prerequisite for overcoming the species barrier. For H2 and H3 HAs, the substitutions HA-Q226L and HA-G228S (all aa positions are referred to H3 numbering) have been described to confer a complete switch from Siaα2,3Gal to Siaα2,6Gal binding (Vines et al., 1998). Furthermore, in H9 and H7 subtypes HA-Q226L substitution also increased Siaα2,6Gal binding *in vitro* (Wan and Perez, 2007, Liu et al., 2014). In the case of H1 HAs, the HA-E190D and the HA-D225G substitutions are critical for the shift from avian to human receptor recognition (Glaser et al., 2005, Stevens et al., 2006). However, the mechanisms that rule the specific aa that determine receptor-binding specificity within the RBS are not completely understood as described mutations vary among the different HA subtypes or even virus strains. Table 2 summarizes the main RBS aa mutations described as important for mammalian adaptation of different HA subtypes.

The HA protein also mediates the fusion of the viral envelope with the endosomal membrane derived from the host cell. This fusion is influenced by the HA heat stability and the pH at which HA experiment the conformational changes within the endosome. Different influenza viruses have different activation pH value ranges, in avian viruses it ranges from 5.5 to 6.0 while in human viruses pH values ranges vary from 5.0 to 5.5 (Russier et al., 2016). Recent studies demonstrated that HA stability played a role in H5N1 virus adaptation in ferrets (Imai et al., 2012, Herfst et al., 2012). In particular the authors found that mutations in the HA RBS that improved human-type receptor binding had an unfavourable effect in HA stability that required compensatory mutations to offset the virus fitness. Those results were further supported by the description of 8 aa substitutions that emerged after adaptation of European avian like H1N1 virus into swine and that induced changes in HA stability (Baumann et al., 2015). Collectively, these findings suggest that the optimum pH for fusion and HA stability may be critical to ensure virus transmission in a new host.

Table 2. Virus mutations in the HA RBS that have been described as key markers for the adaptation of avian influenza A viruses to mammals.

Mutation	Virus subtype	Model used in the study	Effect of the aa substitution on the virus receptor preference	References
A138S	H1N1 H5N1		Mutation present in earliest H1N1 human isolates and in H5N1 subtype after ten passages in ferrets. Increased affinity for Siaα2,6Gal linkages.	Matrosovich et al., 1997 Herfst et al., 2014
E190D/N/V	H1/ H9N2		Crucial for adaptation of H1 subtype to mammals (together with G225D). Important marker for mammalian adaptation of H9N2 (together with Q226L).	Matrosovich et al., 2000 Cauldwell et al., 2014 Lakdawala et al., 2015
N224K	H5N1		Increased the affinity of H5N1 for Siaα2,6Gal (together with Q226L).	Imai et al., 2012
D225G	H1/ H9N2		Crucial for adaptation of H1 subtype to mammals (together with E190D). Related with higher virulence in human patients and with enhanced affinity for Siaα2,3Gal.	Matrosovich et al., 2001 Liu et al., 2010 Lakdawala et al., 2015 Iovine et al., 2015
Q226L	H3N2 H2/ H9N2 H7N9 H5N1		Crucial for adaptation of H3N2 subtype to mammals (together with G228S). Increased the affinity of H5N1, H7N9 and H9N2 for Siaα2,6Gal.	Connor et al., 1994 Matrosovich et al., 2000 Wan and Perez, 2007 Herfst et al., 2012 Imai et al., 2012 Chen et al., 2012 Lam et al., 2013 Liu et al., 2014
S227N	H5N1		Increased Siaα2,6Gal affinity.	Yamada et al., 2006
G228S	H3N2 H2/ H5N1		Enhanced Siaα2,6Gal affinity (together with Q226L).	Connor et al., 1994 Suzuki et al., 2000 Herfst et al., 2012 Chen et al., 2012

*Nonpolar hydrophobic aa: glycine (G), alanine (A), valine (V), leucine (L), phenylalanine (P), tyrosine (Y) and isoleucine (I).
Polar hydrophilic aa: serine (S), threonine (T), asparagine (N), glutamine (Q).
Negatively charged aa: aspartic acid (D), glutamic acid (E).
Positively charged aa: lysine (K), arginine (R), histidine (H).

1.4.2 The role of the neuraminidase

The NA enzymatic activity is essential for the optimal release of the viruses from host cells, as well as for the escape from extracellular inhibitors and the prevention of self-aggregation (Colman, 1994, Yang et al., 2014). Many studies suggested that an optimal balance between the HA and the NA activities is required for efficient virus replication and transmission (Wagner et al., 2002). This balance can be disturbed by reassortment or transmission to a new host with a different set of receptors. For instance, the NA proteins of most influenza viruses have higher enzymatic activity

against Siaα2,3Gal, which may be explained by the need to overcome the Siaα2,3Gal rich mucins in the upper respiratory tract (URT) (Richard et al., 2014). However, the human derived N2 and the pH1N1 N1 NAs showed enhanced activity against Siaα2,6Gal that balances the marked Siaα2,6Gal HA activity (Kobasa et al., 1999, Garcia et al., 2014). Additionally, by passaging reassortant viruses with avian HA and human NA it was shown that replication was reconstituted when mutations occurred in HA and/or NA, which led to a functional balance (Shtyrya et al., 2009). On the other hand, Herfst et al. and Kimble et al. described efficient transmission in ferrets of H5N1 and H9N2 viruses containing purely avian NAs (Herfst et al., 2012, Kimble et al., 2013). These findings suggest that adaptation of NA is not mandatory to achieve mammalian adaptation of AIVs. However, the exact nature of the changes in the NA that are needed to achieve efficient adaptation of AIVs to humans is not fully understood.

1.4.3 The role of the polymerase proteins and the internal genes

The PB2 gene, in particular the aa residue 627, has been appointed as the main polymerase determinant for influenza adaptation to a new host (Subbarao et al., 1993, Cauldwell et al., 2014). Mammalian viruses typically contain PB2-627K whereas avian viruses possess PB2-627E. The presence of PB2-627E restricts replication at 33^{o} C, the temperature of the human URT. (Cauldwell et al., 2014). Scull et al. (2010) found that the PB2-K627E resulted in restricted replication of the virus in human respiratory epithelial cells, regardless of the temperature conditions (32^{o}C versus 37^{o}C). Interestingly, the pH1N1 did not contain this mutation. However, to compensate for the lack of PB2-627K, pH1N1 contains the PB2-Q591K substitution (Moncorge et al., 2013). In addition to those mutations, PB2-D701N was demonstrated to enhance the binding of PB2 to importin α (Moncorge et al., 2010). The importin α is responsible protein for the migration of the PB2 to the nucleus, thus the PB2-D701N substitution improves nuclear localization of the polymerase in mammalian cells.

The complete internal gene cassette has been also demonstrated to be relevant for the adaptation of avian viruses to mammals. In 2009, Sorrell et al. obtained an efficient ferret-to-ferret droplet transmission of the reassortant H9N2 virus containing human-seasonal H3N2 internal genes after ten serial passages in ferrets

(Sorrell et al., 2009). Additionally, since 2011, H3N2v and H1N2v viruses containing a pH1N1-origin M gene have been recurrently isolated from humans (Centers for Disease Control and Prevention, 2016). This finding put the attention on those viruses and their "quadruple-reassortant" internal genes. Moreover, while pH1N1 viruses efficiently transmit among ferrets, a reassortant possessing the North American "triple-reassortant" origin NA and M genes virus isolated from humans did not transmit to all exposed ferrets (Lakdawala et al., 2011). It also suggested that the European "avian-like" swine NA and M genes contributed to the improvement of the transmission of these viruses, at least in the ferret model. This is consistent with a study that demonstrated the importance of the pH1N1 NA and M genes for replication and transmission of H9N2 reassortant viruses in pigs (Ma et al., 2012). In more recent studies performed in pigs and ferrets reassortant viruses containing avian H9N2 or H5N1 HA and NA in the pH1N1 backbone, an improved replication and transmission was demonstrated when compared to the parental viruses (Qiao et al., 2012, Abente et al., 2016, Imai et al., 2012). To sum up, the peculiar combination of internal genes that is present in the pH1N1 may be an advantage for novel HA and NA genes to overcome the species barrier.

1.5 Adaptation of avian influenza viruses to swine

1.5.1 Natural infection of pigs with avian influenza viruses

Infections of pigs with AIVs in nature have been recurrently documented. In fact, "avian-like" H1N1 viruses emerged from birds and became endemic in pigs in Europe and Asia (Lewis et al., 2016). Since 1998, H9N2 influenza viruses have been recurrently isolated from pigs in Hong Kong and China. This subtype is, together with "avian-like" H1N1, the AIV subtype most frequently reported in pigs (Peiris et al., 2001, Yu et al., 2008b, Yu et al., 2011). In addition, recent serological studies in pigs revealed a prevalence from 4.9% up to 15.6% for antibodies against H9N2 viruses in slaughterhouses in China (Li et al., 2015, Wang et al., 2016). H1N1, H3N3 and H4N6 waterfowl-origin influenza viruses were recovered in pig farms in Canada between 1999 and 2002 (Karasin et al., 2000a, Karasin et al., 2000b, Karasin et al., 2004). Although serological data suggested transmission of H4N6 between pigs, the infection was not sustained (Karasin et al., 2000b). In China, an H10N5, probably

originating from ducks was isolated in 1 pig at the slaughterhouse in 2010 (Wang et al., 2012). HPAIV have been also reported to infect pigs in nature. In 2003, while an H7N7 caused outbreaks in poultry in the Netherlands, anti-H7N7 antibodies were detected in pigs housed close to infected poultry farms (Loeffen et al., 2004). The source appeared to be the feeding of the pigs with broken eggs from infected poultry. Furthermore, during the H5N1 outbreaks in South-Eastern Asia, these viruses were isolated from pigs in Indonesia and China (Zhu et al., 2008, Shi et al., 2008, Takano et al., 2009). In addition, serological investigations in Vietnamese slaughterhouses located in the neighbourhood of H5N1 HPAIV reported areas, confirmed a limited number of animals (8 out of 3175) to be seropositive (Choi et al., 2005).

Reassortant viruses containing avian-origin genes have been also isolated from pigs worldwide. Actually, European H3N2 and H1N2 subtypes, North American "triple-reassortant" and pH1N1 viruses contain avian-origin genes (Watson et al., 2015, Lewis et al., 2016). In addition, other reassortant viruses containing avian-origin genes have been isolated from swine worldwide but only as dead-end infections. H3N1 viruses containing human-origin H1N1 (NA), turkey-origin H3N2 (HA and M) and the remaining genes of swine-origin were isolated in North America, 2003 (Lekcharoensuk et al., 2006). Later, in 2006, H2N3 reassortants were also isolated (Ma et al., 2007). In 2008, 2 H5N2 influenza viruses were isolated from pigs in South Korea. The genome of 1 isolate was entirely of avian origin, while the other was a reassortant virus containing swine-origin H3N1 PB2, PA, NP and M genes and the remaining genes from an avian H5N2 virus (Lee et al., 2009). The source of infection of pigs with the AIVs remained unclear in most cases. Only in 2 cases surface water used for watering of the animals was the suspected route for the introduction of the avian viruses into the pigs (Karasin et al., 2000a, Ma et al., 2007).

In most cases the infection of pigs with AIVs resulted subclinical or very mild disease (Karasin et al., 2000a, Karasin et al., 2004, Xu et al., 2004, Ma et al., 2007, Cong et al., 2008, Lee et al., 2009). However, as pointed by the serological studies some natural infections with AIV may be clinically unnoticed in pigs (Peiris et al., 2001, Choi et al., 2005, Wang et al., 2016). So far, most of the wholly AIVs isolated in pigs were not able to establish themselves in swine populations (Guan et al., 2009, Yu et al., 2009),

but the recurrent transmission of those viruses to pigs may increase the risk for the adaptation.

1.5.2 Experimental infections of pigs with avian influenza viruses

Experimental studies also demonstrated the susceptibility of pigs to AIVS. In fact, the first experimental evidence of the susceptibility of pigs to LPAIV was provided in 1981. After intranasal inoculation with several H1N1, H2N3, H3N2 and H7N7 AIVs pigs did not show clinical disease symptoms, but nasal virus shedding was reported for up to 7 days post-inoculation (dpi) (Hinshaw et al., 1981). Those results were confirmed later, in 1994 by Kida and colleagues. Twenty nine (including at least 1 of each HA subtype, from H1 to H13) out of 38 LPAIV from various wild bird species were shed up to 7 dpi and induced a serological response in pigs after intranasal inoculation. Again, the infection was subclinical. These results suggested that pigs could be susceptible to AIVs with non-mammalian HA subtypes. Moreover, it was evidenced that combined infection of pigs with H1N1 SIV and H3N8 AIV resulted in the generation of an H3N1 reassortant with efficient replication in swine (Kida et al., 1994).

Later, since the isolation of H5 and H7 HPAIVs from humans, the susceptibility of swine to those viruses was examined by evaluating whether those viruses may pose a risk for the pig population. For instance, right after the H7N7 HPAIV was isolated from poultry in Belgium and the Netherlands in 2003, Loeffen and colleagues inoculated pigs intranasally with the virus leading to virus replication and shedding. However, pigs did not get sick and the virus did not transmit at all (Loeffen et al., 2004). Similar results were obtained in experimental infections of pigs with H5N1 HPAIVs isolated from Vietnam and Thailand (Choi et al., 2005). Finally, Lipatov and colleagues infected pigs with 4 H5N1 HPAIV isolates from humans and birds from Asia in 2003-2005 (Lipatov et al., 2005). The intranasal inoculation provoked mild weight loss and nasal shedding with 3 out of 4 viruses. On the other hand, intranasal inoculation of pigs with the wholly avian-origin and the avian-swine reassortant H5N2 viruses isolated from pigs in Korea in 2008 resulted in mild symptoms. Both viruses were shed for at least 5 days, but only the reassortant virus was transmitted to contact pigs. This suggests that swine-origin internal genes may give LPAIVs a selective advantage that facilitates pig-to-pig transmission (Lee et al., 2009).

In 2009, the emergence of pH1N1 virus boosted research on the adaptation of AIVs to swine. That year, De Vleeschauwer et al. demonstrated pig susceptibility to different AIV subtypes. However, those viruses did not induce disease and were not transmitted (De Vleeschauwer et al., 2009). In addition, due to their recurrent isolations from humans and pigs, avian H9N2 viruses were also proposed to have the potential to become adapted to pigs. For instance, several *in vivo* and *in vitro* studies demonstrated that those viruses possess internal and surface-gene proteins that are compatible with other subtypes genes. This increases the possibility of the generation of a transmissible virus with a novel HA and NA, to which humans and swine are immunologically naïve. In experimental conditions pigs were also susceptible to H9N2 viruses. Unlike in ferrets the HA-Q226L mutation did not seem to be restrictive (The SJCEIRS H9 Working Group, 2013, Wang et al., 2016). Like in other hosts the wild-type H9N2 influenza viruses showed very limited pig-to-pig transmission. Though other reassortment experiments demonstrated that pH1N1 internal genes increased transmission, it was much less efficient than that of swine adapted H1N1 viruses. Interestingly, reassortant viruses containing H9N2 HA and NA in the backbone of pH1N1 demonstrated higher transmission efficiency when compared to the original H9N2 (Qiao et al., 2012, Obadan et al., 2015). This finding suggests that pH1N1 internal genes may pose an advantage in the adaptation of AIVs to pigs. This hypothesis was also tested in pigs infected with reassortant viruses containing H5N1 HA and NA and pH1N1 internal genes. This reassortant also presented enhanced replication when compared to the parental H5N1. However, the transmission efficiency was still lower when compared to that of endemic SIVs (Abente et al., 2016).

While H7N9 viruses had never been isolated from pigs in nature, those viruses were able to replicate in the swine URT after experimental inoculations. After their introduction in pigs those viruses readily changed, but efficient transmission was never achieved (Zhu et al., 2013, Liu et al., 2014, Siegers et al., 2014, Xu et al., 2014).

In summary, as seen in field, pigs are susceptible to a broad range of AIVs also under experimental conditions. Nevertheless, the changes that are required to make those viruses transmit efficiently between pigs are not known.

1.5.3 The role of the pig in the adaptation of avian influenza viruses to mammals

For a long time, swine have been considered to play a role as a "mixing vessel" for avian and human influenza viruses or as intermediate host for the transmission of AIVs to humans. This hypothesis was first enunciated by Scholtissek and colleagues. It was based on the rescue of a temperature sensitive NP mutant of an avian H7N1 virus by co-infections of chicken embryo fibroblasts with either avian, human or swine H3N2 isolates. They found that the virus could be rescued by all AIVs, by none of the human viruses and by 2 out of 10 SIVs. Thus, they proposed that the NP of SIVs had a broader host range regarding its compatibility in reassortant viruses and that pigs were a potential "mixing vessel" for the generation of those reassortants (Scholtissek et al., 1985). This "mixing vessel" hypothesis was further supported by the subtype similarities between swine and human influenza viruses, the finding that pigs could be simultaneously infected with SIV, AIV and/or human influenza viruses and the frequent isolation of reassortant viruses from pigs (Kida et al., 1994). In 1998, Ito et al. gave molecular support to this hypothesis demonstrating the presence of both Siaα2,3Gal and Siaα2,6Gal receptors in the pig trachea. Furthermore, they demonstrated that some "avian-like" SIV acquired molecular traits of human adaptation by continuous replication in pig tracheal explants (Ito et al., 1998). Therefore, the pig may also act as an intermediate host in which AIVs might gain mammalian adaptation traits. However, later studies demonstrated that the presence of both Sia receptors was restricted to the bronchioles and alveoli and that the distribution of Sia receptors in swine resembles the one of human and ferrets (van Riel et al., 2007, Van Poucke et al., 2010, Nelli et al., 2010, Trebbien et al., 2011).

The emergence of the 2009 pH1N1 virus in swine increased the concern again on pigs as source for pandemic viruses (Smith et al., 2009, Mena et al., 2016). Nowadays, this hypothesis has still not been proven and the exact role of the pig in the generation of pandemic viruses remains unknown. Nevertheless, the pig is an important natural host for influenza A viruses and due to its similarities with humans in terms of anatomy, physiology and immunology they may be an incomparable animal model to better understand the adaptation of AIVs to mammals.

1.6 References

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2 AIMS OF THE THESIS

Influenza A viruses cause influenza or “flu” in humans and few animal species such as birds or pigs. Those viruses are usually species-specific and they have to overcome a strong species barrier to infect new hosts. However, when a virus successfully overcomes that barrier it may pose a significant threat for the novel host species. Birds are considered as a source for influenza viruses that could become adapted to mammals. On the other hand, pigs have been proposed for a long time as intermediate hosts where AIVs can adapt to mammals and become pandemic. However, the mechanisms that rule adaptation of influenza viruses and the possible role of the pig in the generation of novel pandemics are not elucidated yet.

1. To assess whether an avian H9N2 virus could become adapted to pigs by serial passaging and to describe the genetic changes induced by that process. Since 1998, H9N2 AIVs with enhanced binding to human-type receptors have been recurrently isolated from pigs and humans. These isolations increased the public health concern about those viruses. Other research groups already evaluated the possibility of adaptation by serial passaging of H9N2 AIVs into mammalian species such as ferrets and/or guinea pigs. However, the genetic changes required by avian H9N2 influenza viruses to evolve into mammalian transmissible form are not elucidated yet.

2. To evaluate if the introduction of pH1N1 internal genes led to an advantage in the adaptation of H9N2 AIVs to pigs and if this reassortant could reach efficient pig-to-pig transmission. In 2009 the pH1N1 influenza virus emerged and spread worldwide in human and swine populations. This virus was generated in pigs and has unique gene constellation containing genes from avian, human and swine origin. Interestingly, pH1N1 thoroughly reassorted with human and swine endemic viruses generating novel viruses containing pH1N1 internal genes. Furthermore, previous experiments demonstrated that by reassortment with pH1N1 internal genes, H5N1 and H9N2 AIVs enhanced their replication and transmission in ferrets and pigs. However, in pigs those reassortant viruses did not transmit with the same efficiency as the endemic swine influenza viruses.

3 EFFECT OF SERIAL PIG PASSAGES ON THE ADAPTATION OF AN AVIAN H9N2 INFLUENZA VIRUS TO SWINE

Adapted from:

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Abstract

H9N2 avian influenza viruses are endemic in poultry in Asia and the Middle East. These viruses sporadically cause dead-end infections in pigs and humans raising concerns about their potential to adapt to mammals or reassort with human or swine influenza viruses. We performed ten serial passages with an avian H9N2 virus (A/quail/Hong Kong/G1/1997) in influenza naïve pigs to assess the potential of this virus to adapt to swine. Virus replication in the entire respiratory tract and nasal virus excretion were examined after each passage and we deep sequenced viral genomic RNA of the parental and passage 4 H9N2 virus isolated from the nasal mucosa and lung. The parental H9N2 virus caused a productive infection in pigs with a predominant tropism for the nasal mucosa, whereas only 50% lung samples were virus-positive. In contrast, inoculation of pigs with passage 4 virus resulted in viral replication in the entire respiratory tract. Subsequent passages were associated with reduced virus replication in the lungs and infectious virus was no longer detectable in the upper and lower respiratory tract of inoculated pigs at passage ten. The broader tissue tropism after 4 passages was associated with an amino acid residue substitution at position 225, within the receptor-binding site of the hemagglutinin. We also compared the parental H9N2, passage 4 H9N2 and the 2009 pandemic H1N1 (pH1N1) virus in a direct contact transmission experiment. Whereas only 1 out of 6 contact pigs showed nasal virus excretion of the wild-type H9N2 for more than 4 days, all 6 contact animals shed the passage 4 H9N2 virus. Nevertheless, the amount of excreted virus was significantly lower when compared to that of the pH1N1, which readily transmitted and replicated in all 6 contact animals. Our data demonstrate that serial passaging of H9N2 virus in pigs enhances its replication and transmissibility. However, full adaptation of an avian H9N2 virus to pigs likely requires an extensive set of mutations.

3.1 Introduction

AIVs of the H9N2 subtype emerged from the natural waterfowl reservoir and have become endemic in poultry in Asia and the Middle East since the early 1990s (Sun et al., 2010). Genetic characterization and phylogenetic analysis revealed that most poultry H9N2 viruses belong to 1 of 3 different lineages: G1-like, Y280-like and Y439-like, also known as Korean-like (Guo et al., 2000, Slomka et al., 2013). In 1999, H9N2 viruses were for the first time isolated from 2 patients with mild respiratory symptoms (Peiris et al., 1999). These human isolates were genetically and antigenically similar to the G1-like lineage (Lin et al., 2000). Later, from 2002 until 2016, H9N2 viruses belonging to the G1-like and Y280-like lineages have been occasionally reported in humans (Peiris et al., 1999, Butt et al., 2010, World Health Organization, 2016). The first H9N2 virus that was isolated from swine belonged to the Y280-like lineage and was reported in Hong Kong in 1998 (Peiris et al., 2001). Since then, different lineages of H9N2 viruses were sporadically detected in swine in Mainland China (Cong et al., 2007, Yu et al., 2008, Yu et al., 2011). Although these infections of humans and pigs indicate that H9N2 viruses can overcome the species barrier without prior adaptation, no human-to-human or pig-to-pig transmission has been reported so far (Sun and Liu, 2015).

Influenza A virus infection is mediated via the attachment of the viral HA RBS to sialyloligosaccharides present on the host cell surface. Avian influenza viruses preferentially bind sialic acids linked to galactose by an α2,3 linkage (Siaα2,3Gal). Conversely, most human and swine influenza viruses more readily bind to receptors that contain terminal α2,6-linked sialic acid (Siaα2,6Gal) (Matrosovich et al., 1997). Because of the predominant expression of Siaα2,6Gal in the human and swine upper respiratory tract (Van Poucke et al., 2010), a switch from Siaα2,3Gal to Siaα2,6Gal receptor binding preference is considered an important factor for avian influenza virus adaptation to mammals (Matrosovich et al., 2015). Specific aa substitutions in the HA RBS have been associated with enhanced Siaα2,6Gal binding (Taubenberger and Kash, 2010). For H1 subtype viruses, for example, a crucial role has been assigned to the substitution of glutamic acid by aspartic acid at position 190 (HA-E190D) (H3 numbering), and glycine by aspartic acid at position 225 (HA-G225D). For

H2 and H3 subtypes, the crucial changes were substitution of glutamine by leucine at position 226 (HA-Q226L) and glycine by serine at position 228 (HA-G228S) (Connor et al., 1994, Vines et al., 1998, Suzuki et al., 2000,). A significant proportion of poultry H9N2 field isolates contain the HA-Q226L mutation, which has been shown to enhance binding to Siaα2,6Gal and replication of these viruses in human airway epithelial cells *in vitro* (Matrosovich et al., 2001, Wan and Perez, 2007). In addition, Wan *et al*. reported that HA-Q226L increased virus replication and direct contact transmission of H9N2 in ferrets, which are considered the "gold standard" model for human influenza pathogenesis and transmissibility studies (Wan et al., 2008, Matsuoka et al., 2009). However, virus transmission of these H9N2 viruses was still less efficient compared to that of human adapted viruses (Wan et al., 2008). This raises the critical question: which other changes are required to make avian H9N2 viruses fully adapted to a mammalian host?

Pigs are important natural hosts for influenza A viruses. These animals have a similar Sia receptor distribution as humans (Van Poucke et al., 2010), and they are susceptible to both avian and human influenza viruses (Kida et al., 1994, De Vleeschauwer et al., 2009b). Since 1985, pigs have been proposed as intermediate hosts for the adaptation and transmission of avian influenza viruses from birds to humans (Scholtissek et al., 1985, Kahn et al., 2014). However, the exact mechanisms by which AIVs may fully adapt to pigs and cause a pandemic are not completely understood. Previous studies reported on the pathogenicity, infectivity and transmissibility of different H9N2 viruses in swine based on different experimental approaches (Kida et al., 1994, De Vleeschauwer et al., 2009b, Qiao et al., 2012, The SJCEIRS H9 Working Group, 2013, Wang et al., 2016). Furthermore, the infection was subclinical in all cases. Intranasal inoculation of pigs with H9N2 viruses isolated from humans or chickens since the late 1990s resulted in nasal virus excretion for more than 1 day, but transmission was either undetectable or far less efficient than that of endemic swine influenza viruses (Qiao et al., 2012, The SJCEIRS H9 Working Group, 2013, Wang et al., 2016). Serial passaging of AIVs in mammals is a proven strategy to promote the selection of virus variants that are better adapted to replicate in, and transmit between this non-natural host (Sorrell et al., 2009, Herfst et al., 2012, Shichinohe et al., 2013). Here we sought to improve the adaptation of a wild-type

H9N2 AIV by performing ten blind serial passages in influenza naïve pigs. To mimic the natural situation as much as possible, we used virus isolated from the nasal mucosa as inoculum for every subsequent passage in an attempt to select for viruses with improved nasal virus shedding and transmission. By doing so, we obtained an H9N2 virus with a predominant mutation at position 225 in HA that replicated and transmitted better in pigs than the parental virus.

3.2 Materials and methods

Ethics statement

The experiments were authorized and supervised by the Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine of Ghent University, with the EC2013/63 number.

Influenza viruses

A/quail/Hong Kong/G1/1997 (H9N2) was isolated from quail at a live bird market and underwent 7 passages in 11-day-old embryonated chicken eggs, 3 passages in Madin-Darby Canine Kidney cells (MDCK), and 2 passages in pigs. After each pig passage the virus was also passaged once in MDCK cells to grow a large stock to be used in the experiment. The virus stock used in the experiment shares at least 99% identity at the nucleotide and protein levels with the original A/quail/Hong Kong/G1/1997 virus (GenBank accession numbers: AF156435.1, AF156421.1, AF156449.1, AF156378.1, AF156407.1, AF156396.1, AF156463.1 and AF156477.2). This strain was selected because it is representative for the G1-like lineage and contains the HA-Q226L substitution that is known to enhance human-like receptor specificity.

A/California/04/2009 (pH1N1) virus underwent 3 passages in MDCK cells, 1 in eggs and a last passage in MDCK cells before use. This virus is representative of the pH1N1 viruses that circulate worldwide in swine.

Animals

Forty-seven 3-week-old piglets were purchased from a commercial herd that is serologically negative for swine influenza and porcine reproductive and respiratory

syndrome virus. Before the start of the experiments, all animals were free of influenza virus antibodies as demonstrated by a commercial blocking anti-influenza A nucleocapsid ELISA (ID-VET) and by virus neutralization (VN) tests using A/swine/Belgium/1/98 (H1N1), A/swine/Flanders/1/98 (H3N2), A/swine/Gent/7625/99 (H1N2) swine influenza viruses (SIVs), as well as A/California/04/09 (pH1N1). Upon arrival, the animals were housed in a biosafety level-2 (BSL-2) high efficiency particle arresting (HEPA) filtered isolation unit for at least 2 days to allow their acclimatization.

Blind serial passages of avian H9N2 influenza virus in pigs

For the first passage, 2 pigs were housed in a biosafety level-3 (BSL-3) HEPA filtered isolator and inoculated intranasally with 3 ml of phosphate-buffered saline (PBS) containing $10^{6.2}$ $TCID_{50}$ of A/quail/Hong Kong/G1/1997 (H9N2). For inoculation unanesthesized pigs were held in a vertical position with the neck stretched. The inoculum (1.5 ml per nostril) was gradually instilled into the nasal cavity by insertion of a fifteen mm cannula attached to a syringe. To determine virus excretion in the nose, individual pigs were swabbed daily from 0 to 4 days post-inoculation (dpi). At 4 dpi both animals were humanely euthanized by slow injection of an overdose ($\geq$ 100 mg/kg) of pentobarbital in the jugular vein. At the time of necropsy, the following tissue samples were collected for virus titrations: nasal mucosa respiratory part (i.e. nasal turbinates), nasal mucosa olfactory part (*i.e.* ethmoid labyrinth), trachea (upper and lower half) and 5 different samples representative of the entire lung (apical+cardiac lobes left and right, diaphragmatic lobes left and right, and accessory lobe). The nasal mucosae (respiratory part) were pooled and a 20% (w/v) tissue homogenate was prepared and used to inoculate 2 pigs with this passage 1 virus inoculum. Ten blind (meaning that the viral load in the inoculum was not known prior to the infection) serial passages were performed in this way. Nasal swabs from all pigs, tissue samples and the inocula used for the subsequent passages were titrated in MDCK cells.

Swine transmission studies

The inoculum giving the highest virus isolation rates and virus titers throughout the respiratory tract and in nasal swabs was examined for its transmission between pigs.

We compared the transmissibility of mentioned virus to that of the parental H9N2 and that of the pH1N1. Nine 3-week-old pigs were used. At 0 dpi (= 2 days before the start of contact transmission), 3 pigs were housed in 3 separate BSL-3 HEPA filtered isolators (1 index pig per isolator) and intranasally inoculated with $10^{6.5}$ $TCID_{50}$ of the respective virus. Forty-eight hours pi, 2 pigs were introduced in each isolator allowing direct contact with the inoculated pig. All animals were clinically monitored for general (depression and anorexia) and respiratory (coughing, dyspnoea, abdominal thumping, tachypnoea) disease signs, and nasal swabs for virus isolation were collected daily from all pigs during 11 days after cohousing. Sixteen dpi, the animals were relocated to a BSL-2 HEPA filtered isolation unit. Serum samples were collected at 0, 16, 23 and 30 dpi and 0, 14, 21 and 28 days post-contact (dpc).

Growth kinetics of parental and passaged virus

To compare the single-step growth curves of the parental, passaged virus and pH1N1 confluent monolayers of MDCK cells were inoculated with an identical multiplicity of infection of 5 MOI per cell at 37^{o}C. After 1 hour of incubation the cells were washed with phosphate-buffered saline (PBS) containing 10 IU/ml penicillin and 10 µg/ml streptomycin to remove unbound virus particles and overlaid with Minimal Essential Medium (MEM) containing supplements (1 mg/ml lactalbumin, 100 IU/ml penicillin, 10 µg/ml streptomycin, 50 µg/ml gentamycin and 2 µg/ml trypsin). The 3 viruses were tested in triplicate, supernatants were collected at 0, 4, 6, 12 and 24 hours post inoculation and titrated in MDCK cells as described below.

Virus titrations

Cotton swabs were weighed before and after collection to determine virus titers per 100 milligram nasal secretions. Nasal swab samples from both nostrils were suspended in 1 ml PBS supplemented with 10% foetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin and mixed vigorously at 4°C for 1 hour. Tissue samples were weighed and grounded in PBS with 10 IU/ml penicillin and 10 µg/ml streptomycin to obtain 20% (w/v) tissue homogenates. Nasal swab samples and tissue homogenates were clarified by centrifugation and stored at -70°C until titration on MDCK cells. Briefly, confluent monolayers of cells were inoculated with

10-fold serial dilutions of the sample. After 5 days of incubation at 37°C with 5% CO_2, virus positive MDCK cells were visualized by immunoperoxidase staining. The cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature and subsequently incubated with mouse anti-NP monoclonal HB-65 antibody (1:50, ATCC) for 2 hours. Incubation with horseradish peroxidase-conjugated goat anti-mouse polyclonal antibody (1:200, Dako) for 1 hour was followed by a development step with H_2O_2 as substrate and 3-amino-9-ethyl-carbazole (AEC) as precipitating agent. Virus titers were calculated by the method of Reed and Muench (1938) and expressed as $\log_{10}$ 50% tissue culture infective doses ($TCID_{50}$) per 100 milligram (nasal swabs) or per gram (tissues).

Virus neutralization assays

Virus neutralization tests were performed on MDCK cells in 96-well plates, using 100 $TCID_{50}$ of virus per well as previously described (Van Reeth et al., 2003). Briefly, 2-fold serum dilutions were incubated (1h, 37°C) with 100 $TCID_{50}$ of MDCK cell-grown virus. MDCK cells (800.000 cells per ml) were incubated with the virus-serum mixture for 24h, after which virus positive cells were visualized by immunoperoxidase staining.

Data analysis

Because the detection limit was 1.7 $\log_{10}$ $TCID_{50}$ per 100 milligram nasal secrete or gram tissues, samples that tested negative for virus were given a numeric value of 1.6 $\log_{10}$ $TCID_{50}$ per 100 milligram nasal secrete or gram tissues. Samples that tested negative in the virus neutralization assay were assigned a value corresponding to half of the minimum detectable titer. For each transmission experiment, the basic reproduction ratio (R_0) was estimated on the basis of the outcome of the experiment (final-size method) with use of the maximum likelihood estimator. Thus, the most likely reproduction ratio was estimated using the total number of animals in the experiment, the total number of susceptible and infectious animals at the beginning of the experiment, and the number of animals that became infected during the experiment. A pig was considered to be infected when it experienced seroconversion. The 95% confidence intervals (CIs) were constructed symmetrically around the estimated value of R_0 (De Vleeschauwer et al., 2009b).

In addition, nasal virus shedding of individual pigs was quantified by calculation of the area under the curve (AUC). The means of the AUCs were compared between different viruses using standard two–sample Mann-Whitney U tests. Differences were considered significant when $p<0.05$. GraphPad Prism Software, version 5, was used for statistical analyses.

Amino acid sequences alignment

Amino acid sequences of HA proteins from H9N2 viruses isolated from swine and humans in the field were obtained from GenBank. Different human and swine amino acid sequences were compared with the HA of the A/quail/Hong Kong/G1/1997 (H9N2) strain, with special attention to amino acids at positions 190, 225, 226 and 228 because these contribute to transmission between mammals (Matrosovich et al., 1997, Taubenberger and Kash, 2010). Amino acid differences were identified by alignment using MEGA 6.06 software.

RT-PCR

We compared the genetic diversity of the parental wild-type H9N2 virus and 2 samples from the passage 4 virus (which was also selected for the transmission experiments): 1 from the nasal mucosa (upper respiratory tract, due to the limited amount of tissue obtained the virus was amplified in MDCK cells) and another from the lungs (lower respiratory tract). The RNA from these samples was isolated using the QIAamp Viral RNA Mini Kit (Qiagen). cDNA was synthesized with the Transcriptor First Strand Synthesis kit (Roche), as described previously (Van den Hoecke et al., 2015). Two separate reactions were performed, using primers specific for the influenza A vRNAs: CommonUni12G (GCCGGAGCTCTGCAGATATCAGCGAAAGCAGG) and CommonUni12A (GCCGGAGCTCTGCAGATATCAGCAAAAGCAGG). Subsequently, all 8 genomic segments were amplified in 1 PCR reaction using a mix containing an 1:1 mix of CommonUni12G and CommonUni12A cDNA, primer CommonUni13 (GCCGGAGCTCTGCAGATATCAGTAGAAACAAGG) (200 nM) and the Phusion High Fidelity polymerase (Thermo Scientific) (Van den Hoecke et al., 2015, Watson et al., 2013, Zhou et al., 2009). RT-PCR was performed as described (Van den Hoecke et al., 2015), with the first 5 PCR cycles performed with an annealing temperature of 45°C

(instead of 72°C). PCR products were purified using the High Pure PCR Product Purification Kit (Roche) and elution of the DNA was in sterile ultrapure water.

Illumina MiSeq library preparation and sequencing

150 ng of each purified RT-PCR sample was sheared with an M220 focused-ultrasonicator (Covaris) set to obtain peak fragment lengths of 400 bp. The fragment ends of 50 ng of these DNA fragments were repaired using the NEBNext Ultra DNA Library Preparation kit (New England Biolabs), followed by addition of the adaptors by using the NEBNext Multiplex Oligos for Illumina kit (Dual Index Primers Set 1, New England Biolabs). The resulting fragments were size-selected at 300 to 400 bp using Agencourt AMPure XP bead sizing (Beckman Coulter). Afterwards, indexes were added in a limited-cycle PCR (10 cycles), followed by purification on Agencourt AMPure XP beads. Fragments were analysed on a High Sensitivity DNA Chip on the Bioanalyzer (Agilent Technologies) before loading on the sequencing chip. After the 2×250 bp MiSeq paired-end sequencing run, the data were base called and converted to Illumina FASTQ files (Phred + 64 encoding).

Next Generation Sequencing data analysis

Sequence data analyses were performed on the resulting Illumina FASTQ files (Phred + 64 encoding) using CLC Genomics Workbench (Version 7.0.3) following the analysis pipeline as described (Van den Hoecke et al., 2015). The processed sequencing reads were mapped to the consensus sequence obtained after *de novo* assembly of the sequencing reads for the A/quail/Hong Kong/G1/1997 (H9N2) virus stock sample, since this virus stock was used to start the virus adaptation. This consensus sequence is available through GenBank accession number (KY785896-KY785903). The raw sequencing data were submitted to the NCBI Sequence Read Archive where they can be found under project number SRP078326.

Genetic characterization

To evaluate if the mutations described in passage 4 were maintained throughout the subsequent passages, the partial coding sequences of HA, M and PB1 genes were determined for all inocula from passage 5 onwards by direct Sanger sequencing of overlapping RT-PCR products. RNA was extracted from virus with the QIAamp Viral

RNA Mini Kit (Qiagen, Valencia, CA). Reverse transcription and amplification of the genes was done by one-step RT-PCR (One-step reverse-transcription polymerase reaction kit, Qiagen, Valencia, CA) using custom specific primers (primer sequences available upon request). After amplification, RT-PCR products were purified using the Nucleo Spin Gel and PCR clean up (MACHEREY-NA-GEL GmbH&CoKG, Duren, Germany) DNA purification kit. Samples were sequenced by GATC Biotech AG (Constance, Germany) using the Sanger ABI 3730 xl platform. Sequencing was done with custom designed primers (primer sequences available upon request). HA, M and PB1 gene segments were compared at the nucleotide and amino acid (aa) level using MEGALIGN program within the DNASTAR 5.01 software package (DNASTAR, Inc., Madison, WI, USA). The complete genome sequences of A/quail/Hong Kong/G1/1997 (H9N2) were already available from GenBank (accession numbers: AF156378.1 (HA), AF156463.1 (M), AF156421.1 (PB1). The sequences generated in this study are available through GenBank accession numbers (KY785881-KY785895)

3.3 Results

Serial passages of avian H9N2 virus in swine is associated with a transient increase in virus replication

The prime goal of this study was to assess the capacity of an avian H9N2 virus to adapt to swine. For this, ten blind serial passages were performed in influenza naïve pigs. Animals were clinically scored and samples from the upper and lower respiratory tract were isolated to monitor viral loads. None of the pigs showed clinical signs. An overview of the virus titers in nasal swabs of individual pigs through the different passages is shown in Figure 1. H9N2 virus was detected in nasal swabs of all pigs, except for 1 pig during passage 9 and both pigs during passage 10. The highest virus titers were reached during passages 4 and 5 (7.3 $\log_{10}$ $TCID_{50}$/100 mg).

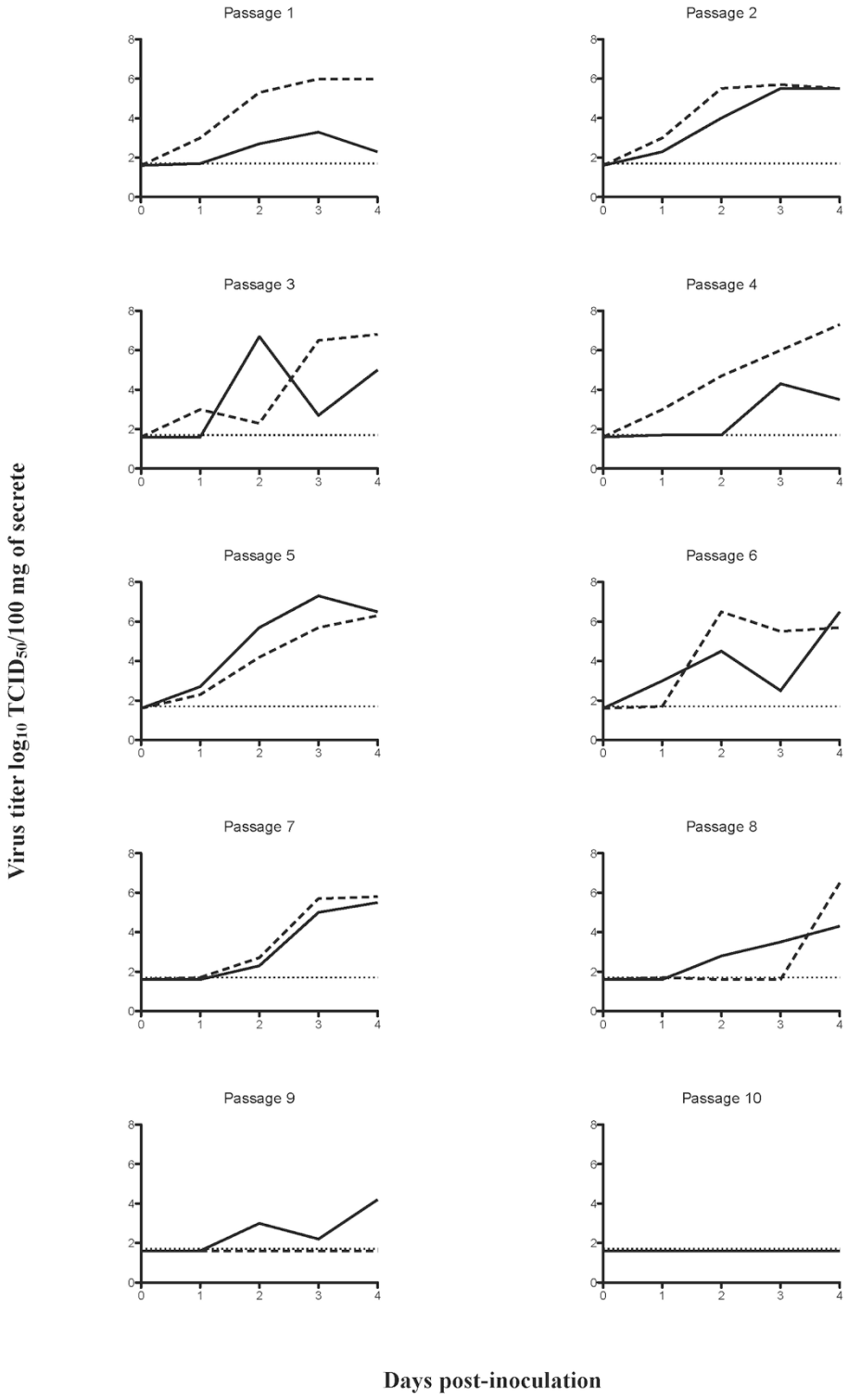

Figure 1. Nasal virus excretion of A/quail/Hong Kong/G1/1997 influenza virus during ten blind serial pig passages. Two pigs (solid line and dashed line) were involved in each passage, each line represents the individual virus titers in nose swabs. The detection limit of the test is indicated with a dotted line at 1.7 $\log_{10}$ $TCID_{50}$/100 mg of secrete.

On day 4 after inoculation, pigs were euthanized and samples from the upper and lower respiratory tract were collected to determine the viral load. Infectious H9N2 virus was found in the respiratory tract of all pigs except for those of passage ten (Table 1). The number of virus positive samples and amount of virus in the different tissue samples were in concordance with nasal virus excretion results, with considerable variation between individual pigs and passages (compare Table 1 and Figure 1). Interestingly, replication rates were higher in the upper respiratory tract (nasal mucosa: respiratory and olfactory parts) with 36 out of 40 (90%) samples testing positive, than in the lower respiratory tract (trachea and lungs) with 65 out of 134 (49%) samples positive. In the upper respiratory tract, virus was isolated at similar frequency from the olfactory and the respiratory parts of the nasal mucosa. After the first 3 passages virus was isolated from some of the lower respiratory tract samples, indicating that at early passages the virus did not replicate uniformly in the entire lung. Only during passage 5, after inoculation with passage 4 virus, infectious virus was clearly detected in all samples, suggesting replication in the entire respiratory tract. Remarkably, following passage 5, the virus seemed to gradually have lost its ability to replicate in the lower respiratory tract and eventually, at passage 10, infectivity was completely lost.

Table 1. Distribution of A/quail/Hong Kong/G1/1997 avian influenza virus in the respiratory tract during ten serial passages in pigs.

Number of passage	Pig number	Virus titer (log_{10} $TCID_{50}$/gram of tissue) at day 4 post-inoculation[a]								
		Nasal mucosa respiratory part	Nasal mucosa olfactory part	Proximal trachea	Distal trachea	Apical+cardiac lobe right	Apical+cardiac lobe left	Diaphragmatic lobe right	Diaphragmatic lobe left	Accessory lobe
1	#1	4	3	2[b]		4.7	<[c]	4.5	<	<
	#2	5.2	6.5	2.5		4.5	<	<	<	1.7
2	#3	5.5	4.5	2		<	<	<	<	<
	#4	6.5	4.2	<		<	<	<	<	2
3	#5	6.5	6.5	<		2	<	<	<	<
	#6	5	5.3	1.7		<	<	<	1.7	1.7
4	#7	7.2	6.2	<	<	<	1.7	<	1.7	<
	#8	5.7	3.3	4.5	4.2	5	1.7	1.7	<	4.3
5	#9	5	5.5	3.3	4.3	5.3	5.2	3.7	5.2	2.3
	#10	7	7.7	5.8	5.3	3.8	2.2	2	5	4.7
6	#11	4.7	5.5	3.7	2.7	4.5	2.6	<	<	5.7
	#12	5.7	4.5	3.5	5.3	5.5	2	4.4	4.4	5.5
7	#13	7	2.8	5.5	<	<	<	<	<	1.7
	#14	5.3	4	2.7	5	6.4	4.7	4.5	3.8	5.7
8	#15	3.7	5	<	<	<	2.3	<	<	<
	#16	5.3	1.7	3.5	5.5	2	<	4.5	3.3	4.7
9	#17	2	1.7	<	<	<	<	<	<	1.7
	#18	4.2	2.3	<	<	<	<	<	<	2.3
10	#19	<	<	<	<	<	<	<	<	<
	#20	<	<	<	<	<	<	<	<	<

[a] Virus titers are shown for each individual pig (#).

[b] Proximal and distal trachea samples were combined during passages 1 to 3.

[c] < detection limit (1.7 log_{10} $TCID_{50}$/gram of tissue).

H9N2 virus becomes contact transmissible after 4 serial passages in pigs

To assess the level of adaptation of the virus after serial blind passages in pigs, 3 different viruses were tested in 3 independent direct contact transmission experiments. As a positive control, we used the A/California/04/2009 (A/Cal/04/09) pH1N1 virus, which is representative of swine-adapted influenza viruses (Vincent et al., 2014, Watson et al., 2015, Lewis et al., 2016). The parental A/quail/Hong Kong/G1/1997 (A/Qa/HK/P0) H9N2 virus served as starting material for the serial passages. The passage 4 H9N2 virus sample (A/Qa/HK/P4) that was used to inoculate animals in passage 5 was also selected for the transmission experiment, because based on virus titers in nasal swabs and respiratory tract samples this virus seemed to have gained the highest swine-adaptation. All 3 viruses were amplified in MDCK cells before inoculation of the index pigs and the transmission experiment was performed in triplicate for each virus.

Figure 2 shows the nasal virus shedding of the inoculated and the co-housed direct-contact animals. All piglets remained clinically healthy, based on clinical observation scores. All inoculated animals excreted virus between days 1 and 8 post-inoculation. Comparison of the AUC revealed lower virus excretion for A/Qa/HK/P0 (18.4) and A/Qa/HK/P4 (19.1) than for A/Cal/04/09 (26.6), although these differences were not statistically significant ($p<0.05$). Of all 9 inoculated animals 5 reached a maximum virus titer of ≥ 6.5 $\log_{10}$ $TCID_{50}$/100 mg of secrete. All inoculated animals developed antibodies against the homologous virus (Table 2). Higher antibody titers were observed in A/Cal/04/09 inoculated pigs than in H9N2 infected animals.

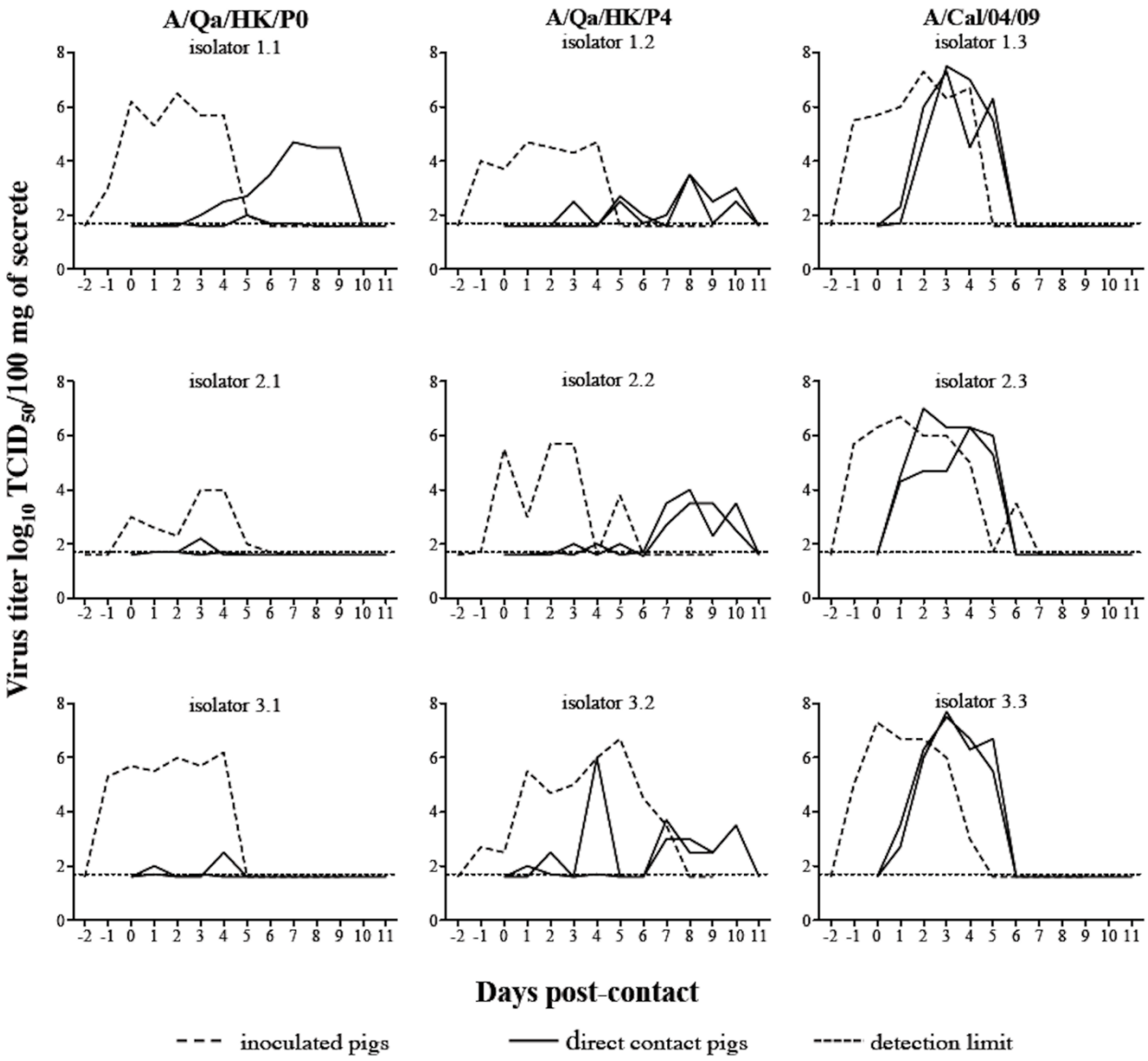

Figure 2. Nasal virus excretion and direct contact transmission of A/Qa/HK/P0, A/Qa/HK/P4 and A/Cal/04/09 influenza viruses in pigs. Three pigs were intranasally inoculated with 6.5 $\log_{10}$ $TCID_{50}$ of the indicated virus per pig and individually housed in different isolators. Forty-eight hours later, 2 direct contact animals were co-housed with the inoculated pigs. Each graphic is identified with a 2 digits number: the first 1 corresponds to the isolator number and the second to the virus tested. Therefore, each column represents a different virus and each row represents a different isolator. The detection limit (dotted line) of the test was 1.7 $\log_{10}$ $TCID_{50}$/100 mg of secrete.

Table 2. Antibody responses in inoculated and direct contact pigs involved in transmission experiments measured by VN test.

Virus strain	**Number of antibody positive pigs** (range of antibody titers)							
	Inoculated pigs (n = 3)				**Direct contact pigs** (n = 6)			
	0	**16**	**23**	**30 dpi**[a]	**0**	**14**	**21**	**28 dpc**[b]
A/Qa/HK/P0	0 (<2)	3 (2-12)	3 (6-24)	3 (6-12)	0 (<2)	2 (24-32)	2 (8-16)	2 (16-24)
A/Qa/HK/P4	0 (<2)	3 (32-384)	3 (64-128)	3 (48-128)	0 (<2)	1 (4)	5 (2-64)	5 (2-12)
A/Cal/04/09	0 (<2)	3 (128-192)	3 (192-384)	3 (512-768)	0 (<2)	6 (192-1024)	6 (256-1536)	6 (192-1024)

[a] dpi: days post-inoculation.
[b] dpc: days post-contact.

Only 1 out of 6 direct contact animals in the A/Qa/HK/P0 group shed virus during more than 5 days resulting in a mean AUC of 2.8. In contrast, all 6 animals that were co-housed with an A/Qa/HK/P4 virus inoculated pig, excreted virus for 5 days or more. The virus excretion was not homogeneous over time (mean AUC of 5.9) and peak viral loads in contact animals were approximately 100-fold lower compared to the viral titers in the nasal excretions of the A/Qa/HK/P4 index pigs (Figure 2). In contrast, all direct contact pigs in the A/Cal/04/09 group shed virus in a pattern that is very similar to that of the inoculated pigs, with a mean AUC of 19.7, and similar peak viral titers. The difference between the AUC values of the A/Qa/HK/P4 direct-contact pigs and the A/Qa/HK/P0 direct contact pigs was not statistically significant ($p<0.05$). However, both groups shed significantly less virus than A/Cal/04/09 contact pigs ($p<0.05$). Five out of 6 A/Qa/HK/P4 contact animals had antibodies against the homologous virus, while only 2 animals that had been exposed to A/Qa/HK/P0 infected index animals seroconverted. As expected, all 6 A/Cal/04/09 contact animals seroconverted, with higher titers than the ones detected in the H9N2 direct contact animals. These data allowed us to determine the R_0 value, a measure for the transmissibility of an infectious agent. R_0 was 0.76 (95% CI: 0.10-5.49) for A/Qa/HK/P0 and 2.27 (95% CI: 0.54-9.29) for A/Qa/HK/P4. Because all A/Cal/04/09 contact pigs became infected, the estimated R_0 value was ∞ (95% CI: 0.83-∞) for this experimental setting. In summary, A/Qa/HK/P4 showed enhanced transmission compared to A/Qa/HK/P0, but both viruses transmitted less efficiency than A/Cal/04/09.

In addition, we evaluated the replication kinetics of the viruses tested in the transmission experiments by determining the single-step growth curve in MDCK cells

for each virus. As demonstrated in figure 3, the growth kinetics of the 3 viruses were very similar. Therefore, the results show that the enhanced *in vivo* transmission of the A/Qa/HK/P4 virus did not have an impact on the *in vitro* replication kinetics of the virus in a continuous cell line.

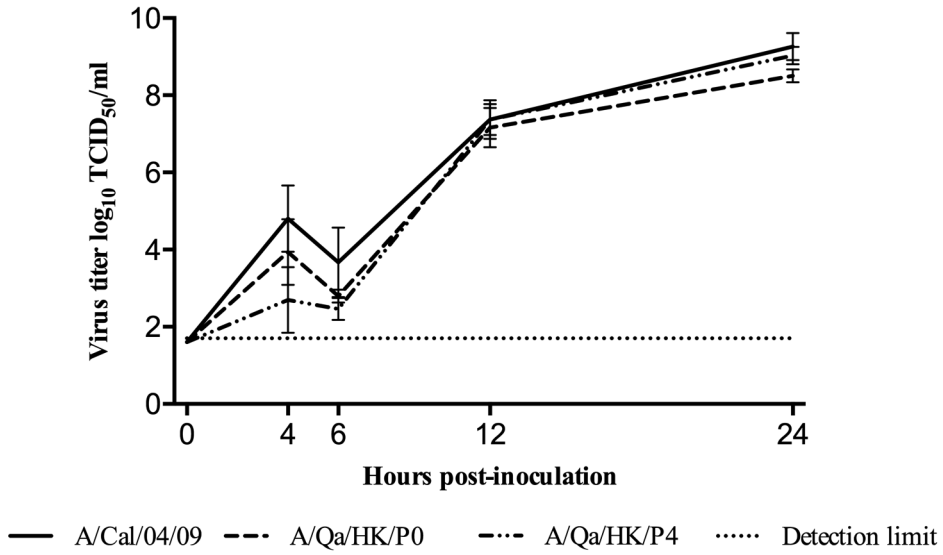

Figure 3. The effect of the serial passaging on the amount of virus produced over the course of the experiment using a single-step growth assay in MDCK cells. Each data point on the curve is the mean ± SD of the 3 independent experiments.

Mutations associated with pig adaptation

We compared the viral diversity present in the H9N2 virus stock that was used to start the serial passages with the viral diversity present in the virus sampled after passage 4 from the nasal mucosa (after a single round of amplification on MDCK cells) and the lung. The viral diversity was analysed in virus (pooled from 2 pigs) sampled after passage 4, since higher virus shedding and higher viral replication in the upper and lower respiratory tract was obtained upon inoculation with this virus. It was anticipated that a comparison of the viral diversity present in the virus from the upper and lower respiratory tract would enable us to determine if these 2 airway compartments favour the acquirement of a different set of mutations for optimal adaptation. In addition, sequencing of the virus stock is needed to determine if the detected pig mutations were already present in the virus inoculum at the start of the

serial passages or if they arose *de novo*. Illumina MiSeq deep sequencing was performed on full genome RT-PCR products of the samples. Nucleotide variants that differ from the consensus sequence of the A/quail/Hong Kong/G1/1997 (H9N2) virus stock sample that was used to start the serial passages and that occur at a frequency of 5% or more are shown in Table 3. Two mutations were present in the starting material. These are non-synonymous mutations that alter 2 adjacent amino acids of the polymerase acidic protein (PA). Interestingly, after 4 passages a substitution of aspartic acid by glycine at position 225 of the HA RBS (HA-D225G) was present in 80.1% of the sequences derived from the viral population of the nasal mucosa and in 99.6% of the viral population of the lung tissue sample. Another substitution was close to 100% present in lung homogenate virus, resulting in an alanine to threonine substitution at position 29 (M2-A29T) in matrix protein 2 (M2). In addition, 2 more substitutions appeared in the nasal mucosa after 4 passages: 1 in PA and another in non-structural protein 1 (NS1). These 2 mutations were present at a frequency below 10%. In the lung virus samples, 3 mutations newly appeared with frequencies ranging from 23.37 to 44.19%. These mutations were present in polymerase basic 1 (PB1), HA and nucleoprotein (NP).

Table 3. Mutations present in parental and passage 4 H9N2 virus isolated from the nasal respiratory mucosa or from the lung. The viral gene segment is indicated, along with the nucleotide position and substitutions, the predicted amino acid change and its position and finally the frequency (percentage) of sequence reads with the detected mutations. Only nucleotide substitutions that resulted in amino acid changes and appeared at a frequency ≥ 5% in the reads are shown. The virus in the nasal mucosa sample was amplified on MDCK cells before sequencing.

Segment	Nucleotide position	Reference	Mutation	Amino acid change	Frequency A/Qa/HK/P0	Frequency A/Qa/HK/P4 (nasal mucosa)	Frequency A/Qa/HK/P4 (lung)
PB1	559	A	G	Glu172Gly	<[a]	<	42.34
PA	1239	G	A	Glu399Lys	<	5.00	<
	1506	A	G	Lys488Glu	10.25	11.21	7.84
	1509	T	A	Cys489Ser	21.37	21.79	10.27
HA	207	A	G	His44Arg	<	<	23.37
	750	A	G	Asp225Gly	<	80.88	99.96
	959	T	C	Phe295Leu	<	<	44.19
NP	1347	G	A	Ala428Thr	<	7.29	10.99
M	818	G	A	M2: Ala29Thr	<	23.26	98.33
NS	191	A	G	NS1: Thr49Ala	<	9.58	<

[a] <: mutation not detected using 5% as variant calling threshold

To evaluate if the substitutions present at the highest frequency in the passage 4 virus (HA-D225G, HA-F295L, M2-A29T and PB1-E172G) were maintained during the subsequent passages we sequenced the HA, M and PB1 coding-genes of the virus present in the inocula used to infect pigs from passages 6 to 10. Interestingly, the substitutions observed in the A/Qa/HK/P4 nasal mucosa were maintained during all passages. However, the substitutions detected in the lung sample were no longer found in further passages.

3.4 Discussion

Avian H9N2 viruses play a pivotal role in the ecology of influenza in poultry in Eurasian countries. Since the late 1990's, recurrent dead-end infections with H9N2 viruses have been reported in pigs and humans (World Health Organization, 2015, Butt et al., 2010, Gu et al., 2014). Moreover, serological studies in Asian poultry workers and in Chinese pigs revealed significant exposure to H9N2 (Blair et al., 2013, Zhou et al., 2014, Wang et al., 2016). The continuous exposure to avian H9N2 viruses might pose a real threat for public health if these viruses would acquire mutations that allow them to transmit efficiently between mammals. Pigs are considered intermediate hosts in which avian H9N2 influenza viruses may acquire such mutations. We have therefore examined whether blind serial passages of an avian H9N2 virus in swine would result in swine-adaptive mutations. We consider an influenza virus as swine-adapted if it succeeds to transmit between pigs in an experimental setting with a similar efficiency as endemic swine influenza viruses. Therefore, we also performed a transmission experiment with the parental and at least partially swine adapted H9N2 virus.

Our results confirm the susceptibility of pigs to intranasal inoculation with avian H9N2 influenza viruses reported in previous experimental studies and in nature (The SJCEIRS H9 Working Group, 2013, Wang et al., 2016, Kida et al., 1994, Sun et al., 2010). However, they are not in agreement with 2 previous studies in which avian H9N2 viruses failed to replicate in pigs. De Vleeschauwer *et al.* (2009) intranasally inoculated pigs with A/chicken/Belgium/818/1979 (H9N2) and found that this strain was not excreted at all by the inoculated pigs (De Vleeschauwer et al., 2009b). Though genetic information of this specific isolate is not available, the lack of replication in the

inoculated pigs was likely due to the fact that European H9N2 isolates belong to the Y439-like lineage, which likely lack the ability to replicate in pigs (Slomka et al., 2013, Wang et al., 2016). In the second study, Qiao *et al.* (2012) used the same A/quail/Hong Kong/G1/1997 (H9N2) strain, to inoculate pigs intratracheally and they detected nasal virus excretion in only 1 out of 12 inoculated animals, albeit at low virus titers (Qiao et al., 2012). The very poor capacity to replicate in swine compared to our findings with A/quail/Hong Kong/G1/1997 (H9N2) can be explained by the different inoculation routes used in our and Qiao's study, which will result in different sites of primary virus replication. It has been demonstrated that replication in the nasal mucosa and nasal virus excretion is secondary to and dependent on replication in the lower airways after intratracheal exposure. Intratracheal inoculation thus usually results in delayed nasal virus excretion and lower nasal virus titers as compared to the intranasal inoculation (De Vleeschauwer et al., 2009a). Moreover, we confirmed previous reports describing the susceptibility of pigs to AIVs of different subtypes (Kida et al., 1994, De Vleeschauwer et al., 2009b, Liu et al., 2014). The parental H9N2 virus replicated in the upper respiratory tract at low to moderate titers while most samples of the lower respiratory tract tested virus negative. The relative lack of Siaα2,3Gal receptors in human and swine upper respiratory tract is thought to restrict the efficient replication of AIVs in mammals. Therefore, a shift from Siaα2,3Gal to Siaα2,6Gal receptor-binding preference has been appointed as a critical step in the adaptation of AIVs to mammalian hosts (Imai and Kawaoka, 2012). For H9, as well as for H3, H5 and H7 avian subtypes, the HA-Q226L substitution contributes to increased Siaα2,6Gal receptor preference and is associated with higher replication rates and transmissibility in pigs and ferrets (Wan and Perez, 2007, Wan et al., 2008, Liu et al., 2014, Van Poucke et al., 2013, Herfst et al., 2012). Though our parental H9N2 virus already contains the HA-Q226L substitution, the virus distribution and replication rates described in our study were similar to those observed in pigs that had been inoculated with H5N2 AIV subtype virus with a "pure" avian genome, i.e. with HA-226Q (De Vleeschauwer et al., 2009a). Our data therefore question the role of the HA-Q226L substitution in replication efficiency in pigs but are in line with more recent studies, which demonstrate that residue 226 in HA is not strictly determinative for the replication of H9N2 viruses in pigs or ferrets (The SJCEIRS H9 Working Group, 2013). Comparative

studies with HA-226L and HA-226Q would be required to evaluate the effect of the HA-Q226L substitution in pigs. In contrast to the parental H9N2 virus, the virus isolated after 4 pig passages replicated homogeneously throughout the entire respiratory tract. We compared pig-to-pig transmission of this virus with that of the parental H9N2 and pH1N1. While the parental H9N2 virus largely failed to transmit to direct contact pigs, the fourth passage virus showed enhanced transmissibility as demonstrated by the AUC and the R_0. This virus showed also higher transmission efficiency when compared with previous studies with H9N2 as well as other avian influenza virus subtypes in pigs. In all of these studies the direct contact pigs excreted no or minimal amount of virus (De Vleeschauwer et al., 2009b, Wang et al., 2016, The SJCEIRS H9 Working Group, 2013). However, transmission of the fourth passage virus remained far less efficient than that of endemic swine influenza viruses. Although the 3 viruses showed different phenotypes in pigs, their growth kinetics in MDCK cells were similar, indicating a poor correlation between the *in vivo* phenotype and *in vitro* replication rates on continuous cell lines. Furthermore, from passage 7 onwards the virus replication progressively decreased until it was lost. This result may be explained by the generation of a narrow bottleneck after passage 4 due to the strong genetic pressure posed by the experimental set up, which may decrease the diversity of the viral population which can hamper the adaptation process (Varble et al., 2014, Zaraket et al., 2015).

Genetic changes during AIV passage in animals are not predictable (Dlugolenski et al., 2011). To better understand the effect of the serial passaging in the virus population and the behaviour of the virus in our transmission experiment we compared the viral diversity in the original wild-type H9N2 virus with the virus isolated from nasal mucosa and lung after 4 passages. We observed that the upper and lower respiratory tract selected for different virus variants. Moreover, the mutations identified in the upper respiratory tract were maintained during the subsequent passages, while those found in the lung virus samples were not maintained in the subsequent passages. Although more genetic analyses might be necessary to better understand this discrepancy between the viral selection pressure in the upper and lower respiratory tract, this points to a possible role of the mutations detected in the lung samples in the enhanced replication and transmissibility. Only 1 out of the 10 mutations we found, the HA-D225G substitution, has been previously described in the literature (Romero-

Tejeda and Capua, 2013, Richard et al., 2014, Taubenberger and Kash, 2010). This mutation arose *de novo* in both the upper and lower respiratory tract after 4 passages and it was associated with a higher replication efficiency during passage 5. Interestingly, this mutation was also present in all 42 swine and 17 human H9N2 field isolates available in GenBank (see Figures 4 and 5). In 1918 and 2009 H1N1 pandemic viruses, HA-D225G substitution has been associated with increased Siaα2,3Gal tropism conferring those viruses dual Siaα2,6Gal and Siaα2,3Gal receptor binding affinity (Lakdawala et al., 2015, Belser et al., 2011, Tumpey et al., 2007). In pigs, as well as in humans, Siaα2,3Gal receptors are predominantly found in the lungs and not in the upper respiratory tract (Van Poucke et al., 2010). The emergence of this mutation after 4 passages could therefore explain the enhanced replication in the lungs. Nevertheless, in *in vitro* sialylglycoprotein binding assays H9N2 viruses containing HA-225G and HA-226L, as our fourth passage virus, showed only slightly increased Siaα2,6Gal binding affinity (Lakdawala et al., 2015, Matrosovich et al., 2001). Though specific binding tests would be needed to clarify the exact role of the HA-D225G substitution, our data suggest that it is an important marker for adaptation of H9N2 AIVs to pigs.

In summary, our results do not reject the theory of the pig as an intermediate host for H9N2 AIVs, but demonstrate that adaptation of these viruses to pigs is a complex process and that the mutations selected after 4 passages in pigs were not sufficient to confer efficient pig-to-pig transmission. It also shows that additional molecular features may be required for efficient transmission of avian H9N2 viruses in pigs. Qiao *et al.* (2012) and Obadan *et al.* (2015) showed that reassortant H9N2 viruses containing pH1N1 internal genes improved replication and transmissibility in pigs, although they were still less efficient than pH1N1 virus. In previous H9N2 adaptation studies in mammals, efficient transmission was only reached with ferret-passaged reassortant H9N2 viruses containing either human seasonal H3N2 or pH1N1 internal genes (Sorrell et al., 2009, Kimble et al., 2011). Therefore, the combination of mammalian adapted internal genes with serial passaging may be required to select a fully transmissible H9N2 virus.

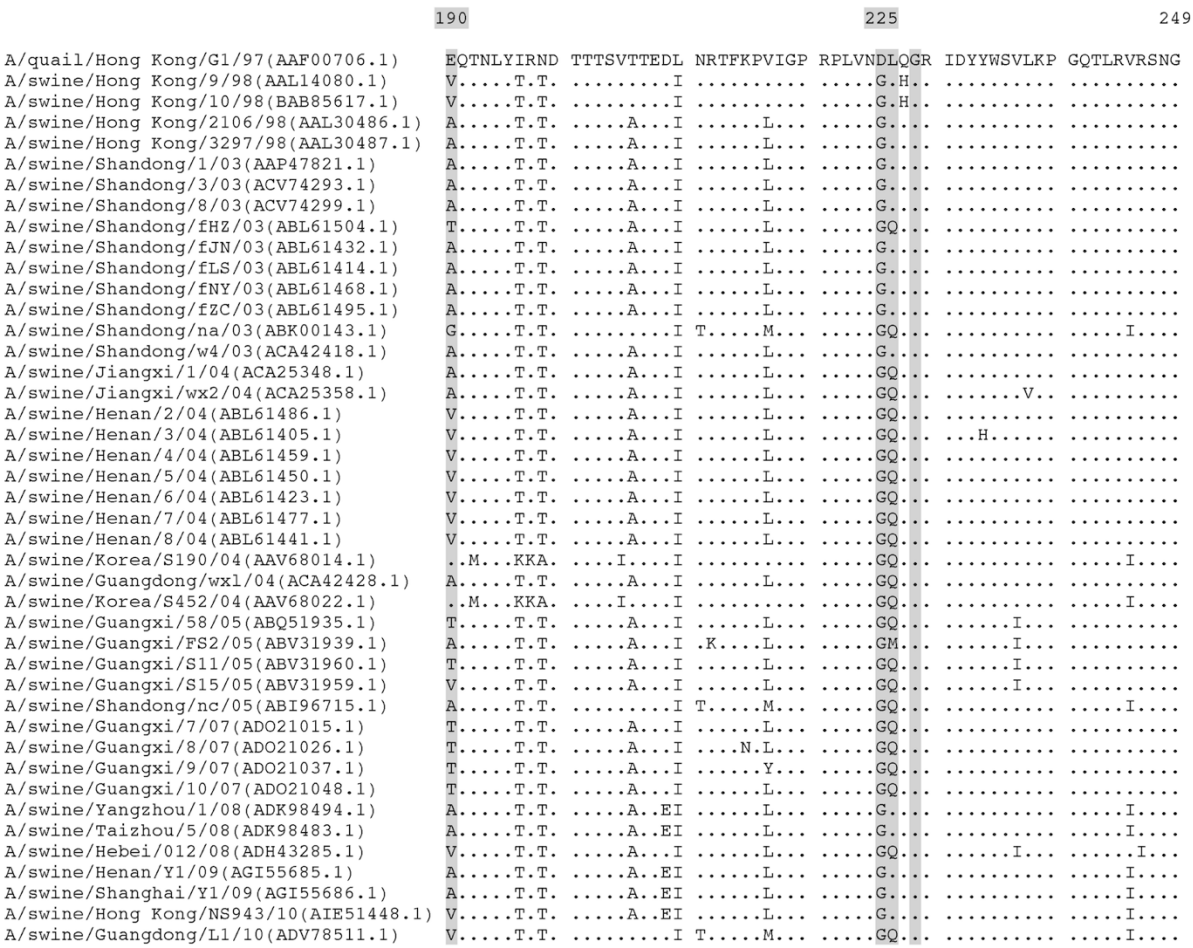

Figure 4. Alignment of the deduced amino acid sequences in the HA of all swine H9N2 isolates (n = 42) available in GenBank on 22nd August 2016. The name of each strain included in the analysis is followed by the accession number between brackets. Residues at positions 190, 225, 226 and 228 are highlighted in gray. Amino acids that are different from those in A/quail/Hong Kong/G1/1997 are shown; conserved residues are shown as dots.

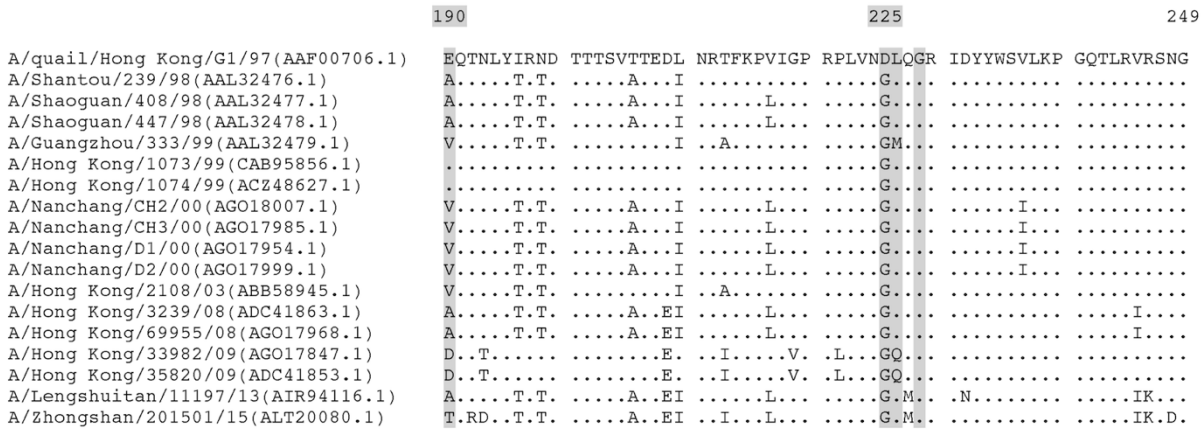

Fig 5. Alignment of deduced amino acid sequences in the HA of all human H9N2 isolates (n = 17) available in GenBank on 22nd August 2016. The name of each strain included in the analysis is followed by the accession number in brackets. Residues at positions 190, 225, 226 and 228 are highlighted in gray. Amino acids that are different from those in A/quail/Hong Kong/G1/1997 are shown; conserved residues are shown as dots.

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4

A REASSORTANT H9N2 INFLUENZA VIRUS CONTAINING 2009 PANDEMIC H1N1 INTERNAL-PROTEIN GENES ACQUIRED ENHANCED PIG-TO-PIG TRANSMISSION AFTER SERIAL PASSAGES IN SWINE

Adapted from:

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Abstract

Avian H9N2 and 2009 pandemic H1N1 (pH1N1) influenza viruses can infect pigs and humans, raising the concern that H9N2:pH1N1 reassortant viruses could emerge. Such reassortant viruses demonstrated increased replication and transmissibility in pig experiments, but were still inefficient when compared to pH1N1. Here, we evaluated if a reassortant virus containing the hemagglutinin and neuraminidase of A/quail/Hong Kong/G1/1997 (H9N2) in the A/California/04/2009 (pH1N1) backbone could become better adapted to pigs by serial passaging. The tropism of the original H9N2:pH1N1 (P0) virus was restricted to the nasal mucosa, with no virus detected in the trachea or the lungs. Nevertheless, after 7 passages (H9N2:pH1N1 (P7)) the virus replicated in the entire respiratory tract. We also compared the transmissibility of H9N2:pH1N1 (P0), H9N2:pH1N1 (P7) and pH1N1. While only 2/6 direct-contact pigs showed nasal virus excretion of H9N2:pH1N1 (P0) for more than 5 days, 4/6 direct-contact animals shed the H9N2:pH1N1 (P7). Interestingly, virus titers in nasal secretions of those 4 animals were similar to those obtained with the pH1N1, which readily transmitted to all 6 contact animals. The broader tissue tropism and the increased post-transmission replication after 7 passages were associated with the HA-D225G substitution. Our data demonstrate that the pH1N1 internal-protein genes together with the serial passages favour avian H9N2 virus adaptation to pigs.

4.1 Introduction

H9N2 avian influenza viruses are currently endemic in poultry in South East Asia and the Middle East (Sun et al., 2010). Since the late 1990s H9N2 viruses have been occasionally isolated from in humans and swine in China, leading to increasing concerns about their pandemic potential (Peiris et al., 1999, Lin et al., 2000, Peiris et al., 2001, Yu et al., 2008, Butt et al., 2010, Wang et al., 2016, World Health Organization, 2016a). However, all reported human or swine H9N2 virus infections were dead-end events and the virus failed to spread within the human or swine population (Sun and Liu, 2015).

A significant proportion of poultry H9N2 field isolates contains a leucine instead of glutamine residue at position 226 in the HA RBS (HA-Q226L, H3 numbering used throughout). This mutation has been shown to enhance HA binding to terminal Siaα2,6Gal and to improve the replication of H9N2 viruses in human airway epithelial cells *in vitro*, supporting concerns about their pandemic potential (Matrosovich et al., 2001, Wan and Perez, 2007, Wan et al., 2008). The binding of the viral HA to sialyloligosacharides, present on the host cell surface, is the first step for influenza virus infection. Because of the predominant expression of Siaα2,6Gal in human and swine upper respiratory tract (Van Poucke et al., 2010, Nelli et al., 2010), most human and swine influenza viruses more willingly bind to receptors that contain Siaα2,6Gal (Matrosovich et al., 1997). In contrast, avian influenza viruses preferentially bind sialic acids linked to galactose by an α2,3 linkage (Siaα2,3Gal). Therefore, a switch from Siaα2,3Gal to Siaα2,6Gal receptor-binding preference is considered an important step for avian influenza virus adaptation to mammals (Matrosovich et al., 2015). Nevertheless, enhanced Siaα2,6Gal binding is not entirely sufficient to make H9N2 viruses transmissible between mammals (Wan et al., 2008, Qiao et al., 2012, The SJCEIRS H9 Working Group, 2013). This suggests that other amino acid substitutions and/or an adequate constellation of internal genes is also required for efficient transmission of H9N2 viruses between mammals.

Due to the segmented nature of the influenza viruses, the RNA segments of 2 (or more) different influenza viruses can “reassort” when co-infecting the same host cell, thereby generating progeny viruses with properties different from the parental viruses (Taubenberger and Kash, 2010). In that sense, the sporadic isolation of avian H9N2

influenza viruses from pigs and humans in China increases the possibility of co-infections with endemic swine or human influenza viruses, which could result in the generation of novel reassortant viruses with enhanced transmissibility in mammals.

The 2009 pandemic H1N1 virus (pH1N1) is a swine-origin reassortant virus containing human, swine and avian influenza virus genes (Smith et al., 2009). After its first appearance in 2009, the virus has spread around the world in both human and swine populations (Brookes et al., 2010). Moreover, novel reassortant viruses containing pH1N1 internal-protein genes and surface-protein genes from other endemic swine influenza virus have been isolated frequently from pigs worldwide (Nelson et al., 2012, Liang et al., 2014, Simon et al., 2014, Vincent et al., 2014). In addition, H3N2 swine influenza viruses containing the pH1N1-derived matrix protein gene (H3N2v) were isolated from 364 human cases in the US since 2011 (Centers for Disease Control and Prevention, 2016). This suggests that pH1N1 internal-protein genes are compatible with several surface-protein gene combinations and allow for robust replication in mammals.

In transmission studies in ferrets, a reassortant H9N2 virus containing pH1N1 internal-protein genes was readily transmitted to all contact animals by respiratory droplets (Kimble et al., 2011). Although similar reassortant viruses showed increased mammalian transmission compared to the parental H9N2, their transmission was still not as efficient as swine-adapted viruses like pH1N1 (Qiao et al., 2012, Obadan et al., 2015). We previously demonstrated that serial passages of A/quail/Hong Kong/G1/1997 (H9N2) virus in pigs mildly enhanced replication and transmission in swine (Mancera Gracia et al., 2017). Although the resulting pig-adapted virus carried a number of mutations compared to the parental virus, transmission was still less efficient than that of naturally circulating viruses (Mancera Gracia et al., 2017). In the present study, we have examined the replication and transmissibility after ten serial pig passages of a reassortant virus containing A/quail/Hong Kong/G1/1997 (H9N2) surface-protein genes and NP within the A/California/04/2009 (pH1N1) background to evaluate if the pH1N1 internal genes confers an advantage in H9N2 adaptation to pigs. This serial passaging of the H9N2:pH1N1 reassortant resulted in a virus that replicated in and transmitted between pigs at high rates. The predominant mutation in the passaged reassortant virus was an aspartic acid to glycine at position 225 in the HA

RBS. Therefore, our results showed that the combination of reassortment and mutations induced by the serial passages generated a virus with a predominant mutation at position 225 in HA RBS that replicated and transmitted at high rates in pigs.

4.2 Materials and methods

Viruses

The reassortant virus containing A/California/04/2009 (pH1N1) internal genes and A/quail/Hong Kong/G1/1997 (H9N2) HA and NA was produced by reverse genetics at the Department of Diagnostic/Pathobiology at Kansas State University. The virus was plaque purified, passaged 3 times in MDCK cells, characterized and experimentally validated (Qiao et al., 2012). Another passage in MDCK cells was made at the arrival of the virus to the Laboratory of Virology at Ghent University to generate a stock to be used in this study.

A/California/04/2009 (pH1N1) was kindly provided by the Centers for Disease Control and Prevention (US CDC). The virus underwent 3 passages in MDCK cells at the US CDC. Later 1 passage in eggs and 1 in MDCK cells were performed in the Laboratory of Virology at Ghent University before use.

Pig passages and transmission studies

Forty-seven 3-week-old piglets were purchased from a commercial herd free of swine influenza and porcine reproductive and respiratory syndrome virus. Upon arrival, pigs were confirmed seronegative to influenza virus with a commercial blocking anti-influenza A nucleocapsid ELISA (ID-VET) and by virus neutralization (VN) tests. Consecutive passages started with the housing of 2 pigs in a biosafety level-3 (BSL-3) HEPA filtered isolator, and their intranasal inoculation with 3 ml of phosphate-buffered saline (PBS) containing $10^{6.5}$ $TCID_{50}$ of virus. Pigs were clinically monitored daily for general (depression, anorexia) and respiratory (coughing, dyspnoea, abdominal thumping, tachypnoea) symptoms. Nasal swabs were also collected daily from 0 to 4 dpi. At 4 dpi both pigs were euthanized with a lethal dose of pentobarbital sodium (≥ 100 mg/kg) in the jugular vein. The following tissue samples were collected for virus

titrations: nasal mucosa respiratory part (i.e. nasal turbinates), nasal mucosa olfactory part (i.e. ethmoid labyrinth), trachea (distal and proximal half) and 5 different samples representative of the entire lung. The nasal mucosae (respiratory part) were pooled and a 20% (w/v) tissue homogenate was prepared and used to inoculate 2 pigs with this passage 1 virus inoculum. Ten blind (meaning that the viral load in the inoculum was not known prior to the infection) serial passages were performed in this way. Nasal swabs from all pigs, tissue samples and the inocula used for the subsequent passages were titrated on MDCK cells.

For each transmission experiment nine 3-week-old pigs were used. At 0 dpi 3 pigs were housed in 3 separate BSL-3 HEPA filtered isolators (1 index pig per isolator) and intranasally inoculated with $10^{6.5}$ $TCID_{50}$ of the respective virus. Two dpi, 2 pigs were introduced in each isolator allowing direct contact with the inoculated pig. All animals were clinically monitored and nasal swabs for virus isolation were collected daily from all pigs during eleven days after cohousing. Sixteen dpi, the animals were relocated to a BSL-2 HEPA filtered isolation unit. Serum samples were collected at 0, 16, 23 and 30 dpi and 0, 14, 21 and 28 days post-contact.

Ethical statement

All experimental procedures were conducted in accordance with E. U. Animal Welfare Directives, and authorized and supervised by the Local Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine of Ghent University (EC2013/63 number).

Virus titrations

Nasal swabs from both nostrils were suspended in 1 ml PBS supplemented with 10% foetal bovine serum, 100 IU/ ml penicillin and 100 µg/ ml streptomycin and mixed vigorously at 4°C for 1 hour. Tissue samples were weighed and grounded in PBS with 10 IU/ml penicillin and 10 µg/ml streptomycin to obtain 20% (w/v) tissue homogenates. Briefly, confluent monolayers of cells were inoculated with 10-fold serial dilutions of the sample. After 5 days of incubation at 37°C with 5% CO_2, virus positive MDCK cells were visualized by immunoperoxidase staining. The cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature and subsequently incubated with mouse anti-NP monoclonal HB-65 antibody (1:50, ATCC)

for 2 hours. Incubation with horseradish peroxidase-conjugated goat anti-mouse polyclonal antibody (1:200, Dako) for 1 hour was followed by a development step with H_2O_2 as substrate and 3-amino-9-ethyl-carbazole (AEC) as precipitating agent. Virus titers were calculated by the method of Reed and Muench (1938) and expressed as $\log_{10}$ 50% tissue culture infective doses ($TCID_{50}$) per 100 milligram (nasal swabs) or per gram (tissues).

Virus neutralization assays

VN tests were performed on MDCK cells in 96-well plates, using 100 $TCID_{50}$ of virus per well as previously described (Van Reeth et al., 2003). Briefly, 2-fold serum dilutions were incubated (1h, 37^{o}C) with 100 $TCID_{50}$ of MDCK cell-grown virus. MDCK cells suspension (800.000 cells per ml) was incubated with the virus-serum mixture for 24h, after which the virus positive cells were visualized by immunoperoxidase staining. Antibody titers were expressed as the reciprocal of the highest serum dilution that completely inhibited virus replication in MDCK cells.

Data analysis

Nasal virus shedding of individual pigs was quantified by calculation of the AUC. The means of the AUCs of different viruses were compared using standard two–sample Mann-Whitney U tests. Differences were considered significant when $p<0.05$. GraphPad Prism Software, version 5, was used for statistical analyses.

RT-PCR

The RNA from the virus samples was isolated using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized with the Transcriptor First Strand Synthesis kit (Roche), already described (Van den Hoecke et al., 2015). Two separate reactions were performed, using primers specific for the influenza A vRNAs: CommonUni12G (GCCGGAGCTCTGCAGATATCAGCGAAAGCAGG) and CommonUni12A (GCCGGAGCTCTGCAGATATCAGCAAAAGCAGG). Subsequently, all 8 genomic segments were amplified in 1 PCR reaction containing an 1:1 mix of CommonUni12G and CommonUni12A cDNA, primer CommonUni13 (GCCGGAGCTCTGCAGATATCAGTAGAAACAAGG) (200 nM) and the Phusion High Fidelity polymerase (Thermo Scientific) (Van den Hoecke et al., 2015, Watson et al.,

2013, Zhou et al., 2009). RT-PCR was performed as described (Van den Hoecke et al., 2015), with the first 5 PCR cycles performed with an annealing temperature of 45°C (instead of 72°C). PCR products were purified using the High Pure PCR Product Purification Kit (Roche) and elution of the DNA in sterile ultrapure water.

Illumina MiSeq library preparation and sequencing

500 ng of each purified RT-PCR sample was sheared with an M220 focused-ultrasonicator (Covaris) set to obtain peak fragment lengths of 400 bp. Next the NEBNext Ultra DNA Library Preparation kit (New England Biolabs) was used to repair the end and to add the Illumina MiSeq-compatible barcode adapters to 100 ng fragmented DNA. The resulting fragments were size-selected at 300 to 400 bp using Agencourt AMPure XP bead sizing (Beckman Coulter). Then, indexes were added in a limited-cycle PCR (10 cycles), followed by purification on Agencourt AMPure XP beads. The fragments were analysed on a High Sensitivity DNA Chip on the Bioanalyzer (Agilent Technologies) before loading on the sequencing chip. After the 2×250 bp MiSeq paired-end sequencing run, the data were base-called and converted to Illumina FASTQ files (Phred +64 encoding).

Next Generation Sequencing data analysis

Sequence data analyses were performed on the resulting Illumina FASTQ files (Phred 64+ encoding) using CLC Genomics Workbench (Version 7.0.3) following the analysis pipeline as described (Van den Hoecke et al., 2015). The processed sequencing reads were subsequently mapped to the *de novo* assembled consensus sequence of the H9N2:pH1N1 (p7) stock virus sequence. This consensus sequence is available through GenBank accession number (KY785904-KY785911). The processed sequencing reads for the H9N2:pH1N1 (P7) stock virus were mapped to a reference genome composed of the H9N2:pH1N1 (P7) stock virus sequence and the A/quail/Hong Kong/G1/1997(H9N2) virus genome. The raw sequencing data were submitted to the NCBI Sequence Read Archive where they can be found under project number: SRP095510.

4.3 Results

Serial passages of a reassortant H9N2 virus resulted in increased virus replication

To evaluate if serial pig infections made reassortant H9N2 viruses better adapted to swine, 10 blind serial passages were performed in influenza naïve pigs. For each passage 2 co-housed animals were intranasally inoculated with $10^{6,5}$ $TCID_{50}$ of H9N2:pH1N1 (P0). To determine nasal virus excretion, individual pigs were swabbed daily from 0 to 4 days post-inoculation (dpi). None of the pigs showed clinical signs of disease. The reassortant H9N2 virus was detected in nasal swabs of all experimentally infected pigs. The highest amount of virus was excreted during passages 3 and 8 (Figure 1). At 4 days after infection, pigs were euthanized and samples from the entire respiratory tract were collected to determine the viral load. Infectious virus was recovered from the respiratory tract of all pigs during all 10 serial passages (Table 1). Replication rates were higher in the upper respiratory tract (nasal mucosa: respiratory and olfactory parts) with all 40 out of 40 (100%) samples testing positive, when compared to the lower respiratory tract (trachea and lungs) with 81 out of 140 (58%) samples testing positive. In the first passage, no virus could be isolated from the lower respiratory tract. In contrast, during passages 2, 3 and 8, the virus was detected in 17 out of 18 (93%) samples, indicating replication in the entire respiratory tract (Table 1).

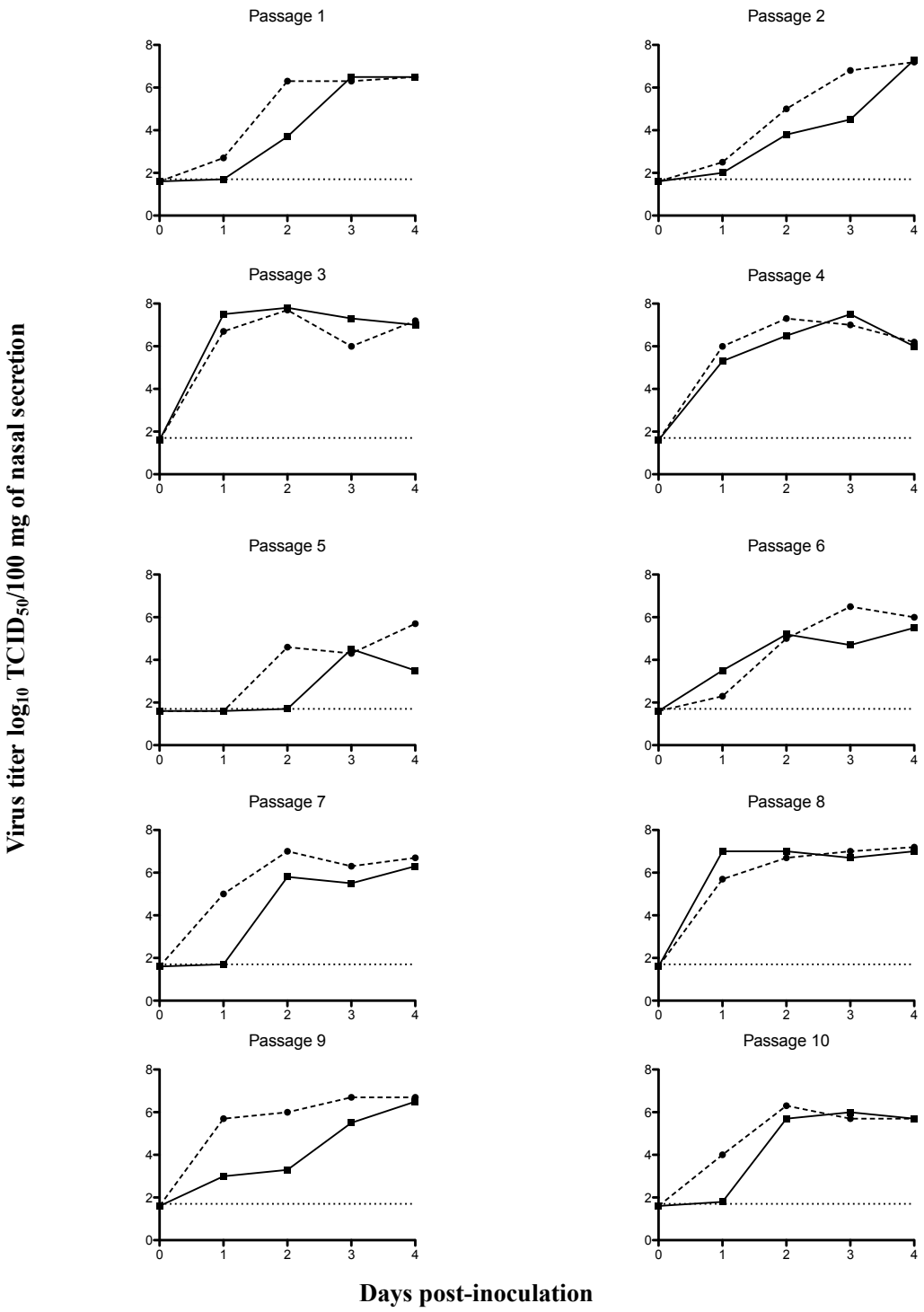

Figure 1. Nasal virus shedding of the reassortant H9N2 virus containing pH1N1 internal-protein genes (H9N2:pH1N1) during 10 blind serial pig passages. Two pigs (solid line and dashed line) were used for in each passage, each line represents the virus titers in nasal swabs of an individual pig. The detection limit of the test is indicated with a dotted line at 1.7 $\log_{10}TCID_{50}$/100mg of secretion.

Table 1. Distribution of reassortant H9N2 virus containing pH1N1 internal-protein genes in the respiratory tract during 10 serial passages in pigs.

Number of passage	Pig number	Virus titer (log_{10} $TCID_{50}$/gram of tissue) at day 4 post-inoculation[a]								
		Nasal mucosa respiratory part	Nasal mucosa olfactory part	Proximal trachea	Distal trachea	Apical+cardiac lobe right	Apical+cardiac lobe left	Diaphragmatic lobe right	Diaphragmatic lobe left	Accessory lobe
1	#1	5.3	5.5	<[b]	<	<	<	<	<	<
	#2	4.5	5	<	<	<	<	<	<	<
2	#3	5.7	5.7	2	4.2	<	3.3	2	3.5	2.3
	#4	7	5.7	4.3	4	4.3	2.5	2.5	3.8	2.5
3	#5	5.7	5.5	5.7	4.7	2.3	3.7	2.3	3	1.7
	#6	5.7	7.3	2.7	2	2.3	4.5	1.7	2.5	<
4	#7	5.5	6.3	2.3	3.8	1.7	<	3.7	<	1.7
	#8	6.3	5.5	5	4.7	5.7	4	4.5	4.5	2.7
5	#9	6	1.7	1.7	<	<	<	<	<	<
	#10	4.7	2.5	4.2	2.7	6	2,8	3.2	<	5.5
6	#11	5.5	3.5	1.7	<	<	<	<	<	<
	#12	6.3	4	<	<	<	<	<	<	<
7	#13	5.7	5.5	3.3	2.5	<	1.7	2.8	<	<
	#14	5.5	4.3	1.7	<	<	<	<	<	<
8	#15	6.3	7	4.3	5.5	5.3	5.5	5.5	5.5	4.5
	#16	6.7	6.3	2.5	2.8	<	3.2	4,8	3,3	4,3
9	#17	6.5	6.7	1.7	<	<	<	1.7	<	<
	#18	6	5.5	2.5	3.5	<	5.5	4.3	3.5	2,7
10	#19	7	5.3	1.7	<	2	<	3.5	2.5	<
	#20	6.8	3.7	1.7	5	5.5	2.2	<	4.7	<

[a] Virus titers are shown for each individual pig (#).

[b] < detection limit (1,7 log_{10} $TCID_{50}$/gram of tissue

After 7 serial passages in pigs, the reassortant H9N2:pH1N1 virus showed enhanced contact transmission

To compare the level of adaptation of the reassortant virus before and after serial passages in pigs, we performed direct-contact transmission experiments with the H9N2:pH1N1 (P0) virus and its descendant isolated after 7 passages in pigs (H9N2:pH1N1 (P7)). The latter virus was chosen because virus titers in nasal swabs and the respiratory tract samples were highest at this passage, suggesting a substantial degree of swine-adaptation. As a positive control, we used a representative swine-adapted virus, the A/California/04/2009 (pH1N1) virus (A/Cal/04/09). All 3 viruses were amplified in MDCK cells before pig inoculation, and the transmission experiment was performed in triplicate for each virus. Figure 2 illustrates the nasal virus shedding of the inoculated and the co-housed direct-contact animals. All animals remained clinically healthy, based on clinical observation. All inoculated pigs excreted virus between days 1 and 7 post-inoculation. A comparison of the averages of the areas under the curve (AUC) revealed a lower virus excretion for H9N2:pH1N1 (P0) (mean AUC 19.4) and H9N2:pH1N1 (P7) (24.0) than for A/Cal/04/09 (26.6), even though these differences were not statistically significant ($p>0.05$). All 3 viruses reached a maximum virus titer ≥ 6.5 $\log_{10}$ $TCID_{50}$/100 mg of secrete. All inoculated animals developed neutralizing antibodies against the homologous virus and these were highest for the A/Cal/04/09 infected pigs (Table 2).

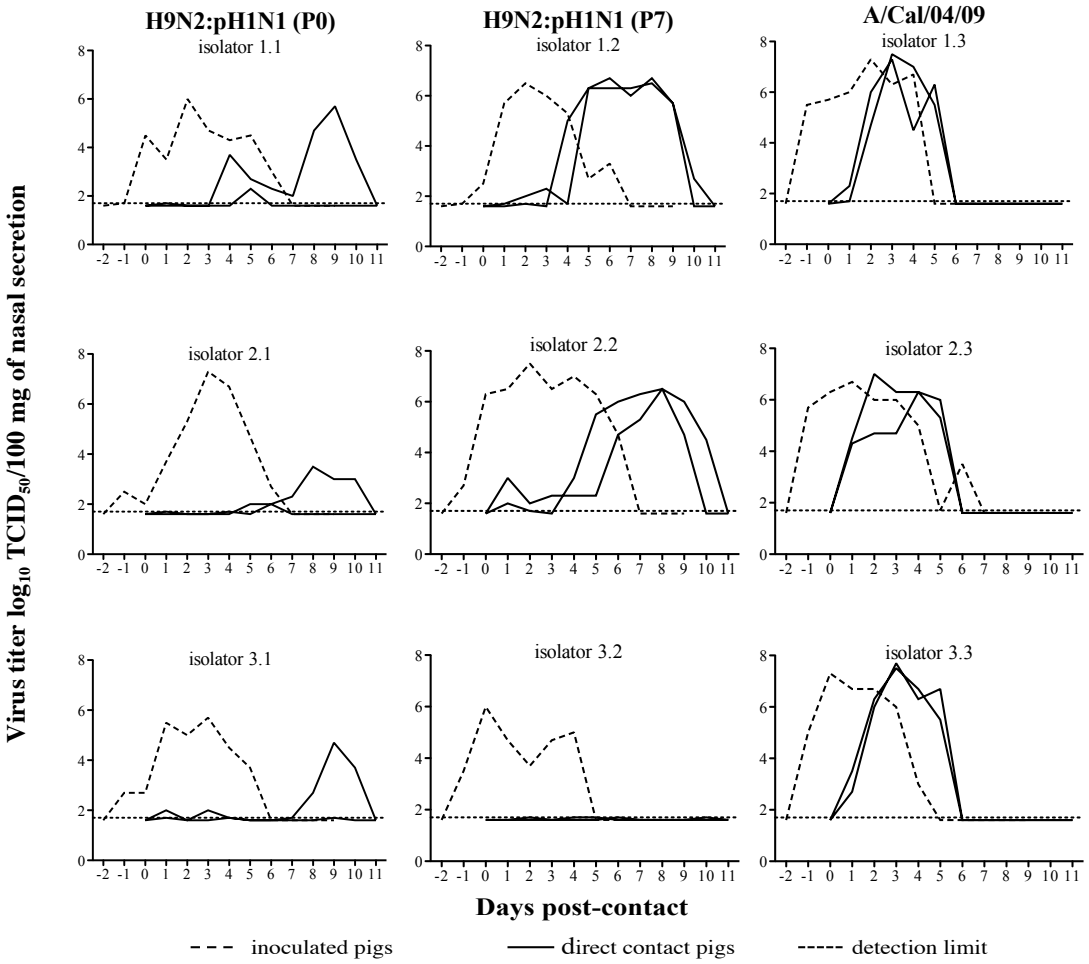

Figure 2. Nasal virus shedding and direct-contact transmission of H9N2:pH1N1 (P0), H9N2:pH1N1(P7) and A/Cal/04/09 influenza viruses in pigs. Three pigs were intranasally inoculated with 6,5 $\log_{10}$ $TCID_{50}$ of the indicated virus and housed in 3 separate isolators. Forty-eight hours later, 2 direct contact animals were co-housed with each inoculated pigs. Each graph is identified with a 2 digits number: the first one corresponds to the isolator number and the second to the virus tested. Therefore, each column represents a different virus and each row represents a different isolator. The detection limit (dotted line) of the test was 1.7 $\log_{10}$ $TCID_{50}$/100 mg of secretion.

Table 2. Serum VN antibody titers in inoculated and direct contact pigs involved in transmission experiments.

	Number of antibody positive pigs (range of antibody titers)							
	Inoculated pigs (n = 3)				**Direct contact pigs** (n = 6)			
Virus strain	**0**	**16**	**23**	**29 dpi**[a]	**0**	**14**	**21**	**28 dpc**[b]
H9N2:pH1N1 (P0)	0 (<2)	3 (16-128)	3 (32-256)	3 (32-192)	0 (<2)	3 (24-192)	6 (12-192)	6 (48-256)
H9N2:pH1N1 (P7)	0 (<2)	3 (48-96)	3 (64-256)	3 (48-128)	0 (<2)	5 (2-256)	4 (96-256)	4 (64-192)
A/Cal/04/09	0 (<2)	3 (128-192)	3 (192-384)	3 (512-768)	0 (<2)	6 (192-1024)	6 (256-1536)	6 (192-1024)

[a] dpi: days post-inoculation.
[b] dpc: days post-contact.

As expected, all 6 A/Cal/04/09 direct-contact pigs excreted high amounts of virus (AUC > 17) and seroconverted with VN titers ≥ 192. While all 6 direct-contact animals in the H9N2:pH1N1 (P0) group also seroconverted and excreted virus, only 2 shed virus with an AUC > 6. In the H9N2:pH1N1 (P7) group, in contrast, just 4 out of 6 direct-contact animals excreted virus but all of them showed an AUC > 21, similar to the A/Cal/04/09 group. The remaining 2 H9N2:pH1N1 (P7) direct-contact pigs did not excrete any virus and failed to seroconvert. VN titers in the 6 H9N2:pH1N1 (P0) and 4 out of 6 H9N2:pH1N1 (P7) direct-contact pigs were < 256.

Mutations associated with enhanced transmission

We next compared the variants present in the H9N2:pH1N1 (P0) virus population and the H9N2:pH1N1 (P7) virus isolated from the nasal mucosa of a directly inoculated pig and the direct-contact pig with the highest nasal excretion in the transmission study. By comparing the virus population from the directly-inoculated and the direct-contact pigs we aimed to determine if the mutations selected during the serial passaging were maintained after transmission or if the transmission itself favoured the acquirement of a different set of mutations. In addition, the H9N2:pH1N1 (P0) virus stock was included to determine whether the detected variants in the passage 7 virus were already present in the virus stock before pig adaptation. Illumina MiSeq deep sequencing was performed on full genome RT-PCR products of the samples. Sequencing of H9N2:pH1N1 (P0) virus revealed contamination of the H9N2:pH1N1 virus stock with H9N2 internal-protein genes, which were most pronounced for the PB1, NS and NP gene segments (see Table 3). Interestingly, passage of the reassortant H9N2:pH1N1 virus stock in pigs resulted in the selection of a virus that had lost these internal-

protein coding H9N2 gene segments, except for the NP segment which was still of H9N2-origin after 7 pig passages. Sequence analysis of the H9N2:pH1N1 (P7) virus after contact transmission showed that the genetic makeup of this virus was nearly identical to the H9N2:pH1N1 (P7) stock virus. The selection for a H9N2 virus with the pH1N1-origin internal protein-coding gene segments suggests a strong genetic bottleneck that leads to elimination of reassortant viruses with H9N2 internal protein-coding gene segments upon viral passaging in pigs. The nonsynonymous variants that were detected at a frequency ≥ 5% in the H9N2:pH1N1 (P7) stock virus and the H9N2:pH1N1 (P7) virus isolated from the direct-contact pig after mapping the sequencing reads to the*de novo* assembled consensus sequence of the H9N2:pH1N1 (P7) stock virusare shown in Table 4. As a consequence of the contamination, the sequencing reads of the H9N2:pH1N1 (P0) stock virus were mapped to a reference genome composed of the *de novo* assembled consensus sequence of the H9N2:pH1N1 (P7) stock virus and the reference sequence for the A/quail/Hong Kong/G1/1997(H9N2) virus. The nonsynonymous variants detected at a frequency ≥ 0.5% in the H9N2:pH1N1 (P7) genome are shown in Supplementary Table S1.

Table 3. Mapping of the reads of the virus stock of H9N2:pH1N1 (P0), H9N2:pH1N1 (P7) and H9N2:pH1N1 (P7) isolated after direct-contact transmission to the reference genome sample obtained from A/quail/Hong Kong/G1/1997 (H9N2) and A/California/04/2009 (pH1N1). The viral gene segment is indicated along with the frequency (percentage) of sequence reads originating from either one of the parental strains.

Virus stock	Segment	Percentage of genes from … virus	
		A/quail/Hong Kong/G1/1997 (H9N2)	A/California/04/2009 (pH1N1)
H9N2:pH1N1 (P0)	PB2	6	94
	PB1	19	81
	PA	11	89
	HA	100	0
	NP	91	9
	NA	100	0
	M	26	74
	NS	91	9
H9N2:pH1N1 (P7)	PB2	0	100
	PB1	0	100
	PA	0	100
	HA	100	0
	NP	100	0
	NA	100	0
	M	0	100
	NS	0	100
H9N2:pH1N1 (P7) direct-contact	PB2	0	100
	PB1	2	98
	PA	0	100
	HA	100	0
	NP	100	0
	NA	100	0
	M	0	100
	NS	0	100

Table 4. Variants present in the passage 7 reassortant (H9N2:pH1N1 (P7)) virus isolated from the nasal mucosa and from the nasal swab of the direct-contact pig with the highest excretion. The viral gene segment is indicated, along with the nucleotide position and substitutions, the predicted amino acid change and its position and the frequency (percentage) of sequence reads with the detected mutations. Only nonsynonymous substitutions with a frequency ≥ 5% in the reads are shown. The virus in the nasal mucosa sample was amplified on MDCK cells before sequencing.

Segment	Nucleotide position	Reference	Mutation	Amino acid change	Frequency H9N2/pH1N1 (P7) (nasal mucosa)	Frequency H9N2/pH1N1 (P7) (nasal swab)
PA	1260	T	A	Trp406Arg	-	5.09
	1990	TA	-	Leu649-	-	5.56
PB2	220	C	G	Thr58Ala	-	18.55
	1651	C	T	Thr535Met	46.74	-
	1891	T	C	Ile615Thr	8.09	-
HA	407	C	T	Leu119Phe	27.52	-
	750	A	G	Asp225Gly	47.43	99.70
NS	582	G	A	NS1: Gly179Gln NS2: Gly22Arg	30.45	-
	862	C	T	NS2: Ala115Val	-	25.83

The H9N2:pH1N1 (P7) stock virus showed variation above 5% at positions 535 and 615 of the basic polymerase 2 protein (PB2), position 119 of the HA and position 179 of the non-structural 1 protein (NS1) (Table 4). Nonetheless, those variants were not maintained after transmission to the contact pig. Interestingly, only 1 mutation associated with an amino acid substitution was selected after 7 passages and maintained after transmission, *i.e.* the substitution of aspartic acid by glycine at position 225 of the HA RBS (HA-D225G). This mutation was absent (Supplementary Table S1) in the H9N2:pH1N1 (P0) virus stock and present in 47.4 % and 99.7 % of the sequences derived from the viral population isolated after 7 passages and the transmitted virus, respectively. Two other mutations appeared *de novo* after transmission at a frequency > 15%: a substitution of alanine to valine at position 115 of the NS1 and a substitution of threonine to alanine at position 58 of the PB2.

4.4 Discussion

Since the late 1990s, avian H9N2 viruses have been isolated from pigs and humans, so far always as dead end events (Butt et al., 2010, Wang et al., 2016, World Health Organization, 2016b). However, the continuous exposure of humans and pigs to avian

H9N2 viruses might pose a real threat for public health if these viruses would acquire the genetic changes that allow them to transmit efficiently between mammals. In this study, we evaluated the efficiency of direct-contact transmission between pigs of a reassortant H9N2 virus containing pH1N1 internal-protein genes, and the effect of serial pig passages on transmission efficiency. We demonstrated, for the first time, that a pig-passaged reassortant H9N2:pH1N1 virus was excreted by more than half of the direct-contact pigs at titers similar to those of the parental pH1N1 virus.

Our data confirm the ability of the H9N2:pH1N1 reassortant viruses to infect pigs as was reported in previous studies (Qiao et al., 2012, The SJCEIRS H9 Working Group, 2013, Obadan et al., 2015). In fact, virus excretion from the nasal cavity and replication in the respiratory tract were present at variable rates during the 10 serial pig passages. This finding contrasts with a previous study from our group using the same parental H9N2 virus (A/quail/Hong Kong/G1/1997), however without reassortment with pH1N1 internal-protein genes (Mancera Gracia et al., 2017). In the latter study, replication and shedding of the parental H9N2 virus was slightly enhanced after 4 pig passages but it was completely lost after 10 passages in pigs. This demonstrates that the presence of the well pig-adapted pH1N1-origin internal-protein genes may pose an advantage for the adaptation of H9N2 virus to pigs. While the H9N2:pH1N1 (P0) virus did not show replication in the lungs during passage 1, it replicated homogeneously in the entire respiratory tract during passage 2. Strikingly, the virus replication in the lungs then progressively decreased during passages 4, 5 and 6 to increase again at passage 7. This suggests the presence of a selection bottleneck due to the genetic pressure generated by the experimental set up (Wilker et al., 2013). However, a deeper analysis of the viral population would be needed to elucidate the possible cause of the decrease in the replication rates.

We consider an influenza virus as swine-adapted when it succeeds to transmit between pigs with a similar efficiency as described for the endemic swine influenza viruses. Therefore, we compared pig-to-pig transmission of the seventh passage virus with that of the original reassortant and pH1N1 viruses. Whereas both reassortant viruses transmitted to direct-contact pigs, the seventh passage virus was clearly shed at higher titers by 4 out of 6 direct-contact pigs. This virus also showed higher

transmission efficiency when compared with previous studies describing H9N2:pH1N1 reassortant viruses in pigs (Qiao et al., 2012, Obadan et al., 2015). In the aforementioned studies ≤ 50% of the direct-contact pigs excreted virus. This higher transmission rate suggests that the serial passaging in pigs contributed to the adaptation of the reassortant H9N2 virus. Yet of the 6 direct-contact pigs 2 animals that were housed in the same isolator did not excrete virus at all. This is most likely due to the low amount of virus excreted by the direct-inoculated pig (AUC 17.1), which not have reached a critical threshold to allow virus transmission. Further detailed genetic analyses of the virus excreted by the donor pig would be needed to better understand if there is, besides the low titer, also a genetic basis for the lack of transmission.

To elucidate the effects of the serial passaging and the pig-to-pig transmission on the virus population, we compared the viral diversity in the original reassortant virus with the virus isolated after 7 passages in pigs and the virus excreted after direct-contact transmission. It should be mentioned here that we detected H9N2 internal-gene segments in our original reassortant virus stock. Interestingly, the serial passaging resulted in the purification of the original virus and, after 7 passages only the NP-coding gene was completely of H9N2-origin. As in the original stock the H9N2-derive NP gene segment was present at 91%, this high percentage might have been the reason for the purification towards the H9N2-origin instead of towards the pH1N1-origin as seen for the other gene segments. However, the NS-coding gene showed the same percentage of contamination and was completely selected upon passaging. Although previous studies demonstrated that pH1N1-origin internal genes conferred an advantage in replication and transmission of influenza viruses in mammals (Mehle and Doudna, 2009, Lakdawala et al., 2011, Kimble et al., 2011, Zhang et al., 2012, Campbell et al., 2014, Abente et al., 2016), those studies focused either on the role of the PB2 and the M-coding genes or on the complete internal gene cassette. Not much is known about the exact role of pH1N1-origin NP-protein gene in the adaptation of influenza viruses to mammals. However, it has been described that the interaction between the NP and importin-α can influence the influenza virus host range. For example, the presence of the NP-N319K substitution was described as an important factor for the replication of H7N7 viruses in mice (Gabriel et al., 2008). Nevertheless,

both the H9N2 and the pH1N1 NP-protein genes lack that mutation. Thus, further analysis with reverse genetic generated reassortants would be necessary to elucidate if the combination of H9N2-origin NP-protein gene and pH1N1-origin internal genes has an effect in the replication and transmission of influenza viruses in mammals. Regarding the mutations detected during the analysis, only 1 of the mutations that appeared in the virus after 7 passages and was maintained even after transmission, the HA-D225G substitution in the RBS. This mutation was therefore likely associated with the higher replication and transmission efficiency of the H9N2:pH1N1 (P7) virus. Interestingly, this mutation was present in all 42 swine and 17 human H9N2 field isolates available in GenBank in November 2016. This mutation was also associated with enhanced replication and transmission of parental H9N2 after 4 passages in pigs (Mancera Gracia et al., 2017). In the 1918 and 2009 pandemic H1N1 viruses this mutation has been associated with increased Siaα2,3Gal tropism (Belser et al., 2011, Lakdawala et al., 2015). In pigs and humans Siaα2,3Gal receptors are mainly found in the lungs (Van Poucke et al., 2010). The presence of the HA-D225G mutation after 7 passages may therefore explain the consistent replication in the lungs during passage 8. Although specific receptor-binding tests would be necessary to elucidate the exact role of the HA-D225G mutation within the HA-RBS, our data suggest that it may be a marker for adaptation of H9N2 viruses to swine.

In line with previous studies (Qiao et al., 2012, Obadan et al., 2015), this report demonstrated that avian H9N2 influenza viruses could reassort with pH1N1 internal genes, resulting in enhanced virus transmission in pigs when compared to the parental, non-reassorted H9N2 virus. The present study also underscores that repeated introduction of reassortant H9N2 viruses into the swine or humans might result in the selection of virus variants with transmission efficiency close to that of the endemic swine or human influenza viruses. However, the lack of transmission detected in 1 of the contact groups used for transmission of passage 7 virus, emphasizes the complexity of the adaptation process of an avian virus to a mammalian species and the need for additional research to better understand this crucial step in cross-species transmission of influenza viruses.

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Author contributions

K.V.R. and J.C.M.G. designed research; J.C.M.G. performed the animal experiments; J.C.M.G. and S.V.D.H. performed the laboratory and the genetic analysis; K.V.R., J.C.M.G., S.V.D.H. and X.S. analysed data; K.V.R., J.C.M.G., S.V.D.H., X.S., W.M. and J.A.R. wrote the paper.

Competing financial interests

The authors declare no competing financial interest.

4.6 References

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Supplementary Table S1: Variants present in H9N2:pH1N1 (P0) virus stock. The sequencing reads of the H9N2:pH1N1 (P0) stock virus were mapped to a combined reference genome composed of the *de novo* assembled consensus sequence of the H9N2:pH1N1 (P7) stock virus and the reference sequence for the A/quail/Hong Kong/G1/1997(H9N2) virus. The nonsynonymous variants detected in the H9N2:pH1N1 (P7) genome above 0.5% are shown per genome segment.

PB2 (H1N1)				
Reference Position	Reference	Allele	Frequency	Amino acid change
81	T	C	0.53	PB2:p.Ser12Pro
90	C	A	0.61	PB2:p.Arg15Ser
141	A	-	0.67	PB2:p.Lys32fs
145	A	G	1.16	PB2:p.Lys33Arg
175	C	A	0.69	PB2:p.Pro43His
196	T	A	0.87	PB2:p.Met50Lys
199	T	-	0.82	PB2:p.Met51fs
214	C	A	0.57	PB2:p.Pro56Gln
237	A	T	0.51	PB2:p.Met64Leu
242	C	A	0.80	PB2:p.Asp65Glu
242	C	G	0.55	PB2:p.Asp65Glu
244	T	C	0.55	PB2:p.Met66Thr
325	C	A	0.53	PB2:p.Pro93His
1651	C	T	100.00	PB2:p.Thr535Met
1933	G	A	11.11	PB2:p.Ser629Asn
1987	TACTGG	-	56.89	PB2:p.Ile647_Val649delinsIle
1993	T	-	3.36	PB2:p.Val649fs
2100	G	T	0.64	PB2:p.Gly685Trp
2101	G	T	0.62	PB2:p.Gly685Val
2138	G	T	0.51	PB2:p.Leu697Phe
2146	A	C	0.76	PB2:p.Glu700Ala
2157	-	AT	7.05	PB2:p.Tyr704fs
2232	G	T	0.54	PB2:p.Gly729Trp
2233	G	T	0.67	PB2:p.Gly729Val
2263	G	T	0.60	PB2:p.Arg739Leu

PB1 (H1N1)				
Reference Position	Reference	Allele	Frequency	Amino acid change
74	AAAAATTCCAGCGCA	-	1.25	PB1:p.Leu10_Gln15delinsLeu
112	C	A	0.67	PB1:p.Pro23His
186	C	A	0.53	PB1:p.Gln48Lys
190	ACT	GAA	13.38	PB1:p.Tyr49_Ser50delins*Thr
192	T	-	4.14	PB1:p.Ser50fs
192	T	A	12.87	PB1:p.Ser50Thr
205	A	G	55.27	PB1:p.Lys54Arg
269	G	T	6.88	PB1:p.Glu75Asp
2146	C	A	0.60	PB1:p.Pro701His
2187	G	A	0.87	PB1:p.Val715Met
2224	G	T	0.63	PB1:p.Arg727Met

Supplementary Table S1: Variants present in H9N2:pH1N1 (P0) stock virus (Continued).

PA (H1N1)				
Reference Position	Reference	Allele	Frequency	Amino acid change
117	G	T	0.52	PA:p.Gly25Trp
118	G	T	0.60	PA:p.Gly25Val
211	A	T	5.97	PA:p.Glu56Val
214	G	T	1.31	PA:p.Arg57Leu
247	C	A	1.23	PA:p.Pro68Gln
340	G	T	0.65	PA:p.Gly99Val
418	G	T	0.60	PA:p.Arg125Met
695	A	C	70.88	PA:p.Gln217His
938	G	T	1.82	PA:p.Glu298Asp
1260	T	A	2.67	PA:p.Trp406Arg
1696	G	T	0.54	PA:p.Arg551Met
1775	G	T	0.76	PA:p.Trp577Cys
1921	G	T	0.54	PA:p.Arg626Met
1922	G	T	0.68	PA:p.Arg626Ser
1944	G	T	0.56	PA:p.Gly634Trp
1957	G	A	1.28	PA:p.Arg638Lys
2062	G	T	0.68	PA:p.Arg673Met
2094	G	T	0.55	PA:p.Gly684Trp
2095	G	T	0.61	PA:p.Gly684Val
2095	GG	AA	0.69	PA:p.Gly684Glu
2097	G	T	0.55	PA:p.Gly685Trp
2107	A	G	0.82	PA:p.Glu688Gly
2140	G	T	0.59	PA:p.Trp699Leu

HA (H9N2)				
Reference Position	Reference	Allele	Frequency	Amino acid change
180	C	A	0.56	HA:p.Pro43His
254	C	A	0.62	HA:p.Leu68Ile
300	C	A	0.76	HA:p.Pro83His
314	C	A	0.53	HA:p.Leu88Met
381	C	A	0.59	HA:p.Pro110His
383	G	T	0.57	HA:p.Gly111Trp
459	C	A	0.54	HA:p.Pro136Gln
575	C	A	0.58	HA:p.Gln175Lys
591	G	T	0.57	HA:p.Arg180Met
616	G	T	0.51	HA:p.Trp188Cys
630	C	A	0.67	HA:p.Pro193Gln
757	G	T	0.75	HA:p.Gln235His
821	G	T	0.74	HA:p.Gly257Trp
899	A	G	3.12	HA:p.Ser283Gly
996	C	A	0.60	HA:p.Pro315His
1158	G	T	0.63	HA:p.Gly369Val

Supplementary Table S1: Variants present in H9N2:pH1N1 (P0) stock virus (Continued).

HA (H9N2)				
Reference Position	Reference	Allele	Frequency	Amino acid change
1437	G	T	0.55	HA:p.Arg462Met
1589	G	T	0.68	HA:p.Gly513Trp
1590	G	T	0.57	HA:p.Gly513Val
1669	G	T	0.56	HA:p.Met539Ile
NP (H9N2)				
Reference Position	Reference	Allele	Frequency	Amino acid change
321	G	T	0.62	NP:p.Gly86Trp
331	C	A	0.54	NP:p.Pro89Gln
376	G	T	0.62	NP:p.Trp104Leu
525	C	A	0.51	NP:p.Leu154Ile
547	C	A	0.54	NP:p.Pro161His
579	C	A	0.62	NP:p.Leu172Ile
583	C	A	0.83	NP:p.Pro173Gln
617	G	T	0.65	NP:p.Lys184Asn
624	G	T	0.54	NP:p.Gly187Trp
632	G	T	0.51	NP:p.Met189Ile
747	G	T	0.70	NP:p.Gly228Trp
810	G	T	0.56	NP:p.Gly249Trp
943	G	T	0.73	NP:p.Arg293Met
948	G	T	0.64	NP:p.Gly295Trp
1041	A	G	1.50	NP:p.Ser326Gly
1110	G	T	0.66	NP:p.Gly349Trp
1111	G	T	0.57	NP:p.Gly349Val
1355	G	T	0.62	NP:p.Lys430Asn
1356	G	T	0.53	NP:p.Gly431Trp
1442	G	T	0.51	NP:p.Gln459His
1443	G	T	0.73	NP:p.Gly460Trp
1447	G	T	0.72	NP:p.Arg461Leu
NA (H9N2)				
Reference Position	Reference	Allele	Frequency	Amino acid change
167	C	A	0.69	NA:p.Pro43Gln
328	C	A	0.75	NA:p.Pro97Thr
329	C	A	0.61	NA:p.Pro97His
367	G	T	0.59	NA:p.Gly110Trp
368	G	T	0.57	NA:p.Gly110Val
377	G	T	0.90	NA:p.Trp113Leu
522	G	T	0.51	NA:p.Leu161Phe
540	G	T	0.69	NA:p.Leu167Phe
619	G	T	0.53	NA:p.Gly194Trp
620	G	T	0.71	NA:p.Gly194Val
811	G	T	0.62	NA:p.Gly258Trp

Supplementary Table S1: Variants present in H9N2:pH1N1 (P0) stock virus (Continued).

NA (H9N2)				
Reference Position	Reference	Allele	Frequency	Amino acid change
878	C	A	0.62	NA:p.Pro280His
1046	G	T	0.61	NA:p.Arg336Met
1066	G	T	0.54	NA:p.Gly343Trp
1084	G	T	0.62	NA:p.Gly349Trp
1119	G	T	0.56	NA:p.Met360Ile
1166	G	T	0.61	NA:p.Arg376Met
1318	G	T	0.59	NA:p.Gly427Trp
1415	G	T	0.56	NA:p.Gly459Val
M (H1N1)				
Reference Position	Reference	Allele	Frequency	Amino acid change
92	C	A	0.69	M1:p.Pro16Gln
103	C	A	0.65	M1:p.Leu20Ile
301	G	T	0.52	M1:p.Gly86Trp
307	G	T	0.51	M1:p.Gly88Trp
308	G	T	0.51	M1:p.Gly88Val
314	C	A	0.57	M1:p.Pro90Gln
366	A	G	0.77	M1:p.Ile107Met
450	G	T	0.62	M1:p.Met135Ile
514	T	G	0.52	M1:p.Ser157Ala
670	A	G	0.66	M1:p.Thr209Ala
687	T	G	0.84	M1:p.His214Gln
703	G	T	0.52	M1:p.Gly220Trp
745	C	A	1.30	M1:p.Leu234Ile
762	C	T	1.29	M2:p.[Pro10Leu]
771	G	A	1.15	M2:p.[Ser13Asn]
774	A	G	1.20	M2:p.[Glu14Gly]
777	G	T	0.70	M1:p.[Met244Ile] M2:p.[Trp15Leu]
780	A	G	1.12	M2:p.[Glu16Gly]
814	CA	TG	1.19	M2:p.Val27_Ile28delinsValVal
825	A	G	1.64	M2:p.Asn31Ser
833	G	T	0.53	M2:p.Gly34Trp
860	AC	CT	1.47	M2:p.Thr43Leu
951	G	T	0.64	M2:p.Arg73Met
963	AA	GG	1.02	M2:p.Gln77Arg
978	G	A	1.13	M2:p.Ser82Asn
NS (H1N1)				
Reference Position	Reference	Allele	Frequency	Amino acid change
61	CA	TG	3.90	NS1:p.[Thr5_Met6delinsThrVal] NS2:p.[Thr5_Met6delinsThrVal]
98	A	G	2.75	NS1:p.Ile18Val

Supplementary Table S1: Variants present in H9N2:pH1N1 (P0) stock virus (Continued).

NS (H1N1)				
Reference Position	Reference	Allele	Frequency	Amino acid change
119	A	C	1.63	NS1:p.Asn25His
127	G	T	0.75	NS1:p.Leu27Phe
138	C	A	0.55	NS1:p.Pro31Gln
161	G	A	0.74	NS1:p.Asp39Asn
177	A	G	0.82	NS1:p.Lys44Arg
186	G	A	0.75	NS1:p.Gly47Asp
189	A	G	0.84	NS1:p.Asn48Ser
222	T	G	0.77	NS1:p.Leu59Arg
235	A	T	0.76	NS1:p.Gln63His
244	AT	GC	0.74	NS1:p.Glu66_Trp67delinsGluArg
272	A	G	0.58	NS1:p.Thr76Ala
329	C	A	0.75	NS1:p.Leu95Ile
342	C	A	0.66	NS1:p.Ser99
355	C	A	0.73	NS1:p.Phe103Leu
364	G	T	0.51	NS1:p.Met106Ile
477	-	C	0.95	NS1:p.Leu144fs
502	G	T	2.33	NS1:p.Glu152Asp
505	G	T	0.52	NS1:p.Glu153Asp
507	G	A	0.56	NS1:p.Gly154Glu
557	T	G	1.07	NS1:p.[Tyr171Asp]
559	T	C	1.11	NS2:p.[Met14Thr]
578	G	A	1.51	NS1:p.[Val178Ile]
582	G	T	0.57	NS1:p.[Gly179Val] NS2:p.[Gly22Trp]
612	G	A	1.30	NS1:p.[Gly189Asp] NS2:p.[Val32Ile]
619	G	A	1.16	NS2:p.[Arg34Gln]
636	A	C	1.00	NS1:p.[Asn197Thr] NS2:p.[Ile40Leu]
638	A	C	0.93	NS1:p.[Ile198Leu]
650	G	A	1.23	NS1:p.[Ala202Thr]
660	A	G	1.08	NS1:p.[Asn205Ser] NS2:p.[Thr48Ala]
662	T	A	0.87	NS1:p.[Cys206Ser]
674	G	T	0.78	NS1:p.[Gly210Trp] NS2:p.[Met52Ile]
680	C	T	1.48	NS1:p.[Pro212Ser]
683	T	C	1.40	NS1:p.[Ser213Pro]
688	A	C	1.04	NS2:p.[Tyr57Ser]
695	G	A	1.30	NS1:p.[Glu217Lys]
697	G	A	1.25	NS2:p.[Ser60Asn]
704	T	C	1.02	NS1:p.[220Arg]

Supplementary Table S1: Variants present in H9N2:pH1N1 (P0) stock virus (Continued).

NS (H1N1)				
Reference Position	Reference	Allele	Frequency	Amino acid change
706	A	G	1.05	NS1:p.[220Trp] NS2:p.[Glu63Gly]
726	G	A	1.02	NS2:p.Gly70Arg
742	A	G	0.91	NS2:p.Glu75Gly
765	A	G	1.03	NS2:p.Met83Val
861	G	A	0.74	NS2:p.Ala115Thr

5 GENERAL DISCUSSION

Potential of adaptation of avian H9N2 influenza viruses to pigs

Avian H9N2 influenza viruses are widespread in land-based poultry in most Eurasian countries (Sun and Liu, 2015). Since 1998, those viruses have been repeatedly isolated from pigs and humans, but always as dead-end events (Guo et al., 2000, Yu et al., 2011, World Health Organization, 2017). However, the continuous exposure to H9N2 AIVs might pose a threat if those viruses could be transmitted efficiently among mammals. Although pigs are considered to play a role as intermediate hosts for the transmission of AIVs to humans, it remains unknown whether H9N2 AIVs could ever become adapted to pigs. Six studies by other researchers already demonstrated that pigs are susceptible to different H9N2 virus strains, but they did not answer the question as to whether H9N2 viruses might become established in swine (Kida et al., 1994, Qiao et al., 2012, The SCEIRS H9N2 working group, 2013, Obadan et al., 2015, Wang et al., 2016). Therefore, the first aim of this thesis was to evaluate the potential of H9N2 viruses to become adapted to pigs.

Our research confirms the ability of H9N2 AIVs to infect pigs as reported in previous experimental studies and in the field (Kida et al., 1994, Sun et al., 2010, The SJCEIRS H9 Working Group, 2013, Wang et al., 2016). The susceptibility of pigs to diverse AIV subtypes has been demonstrated by several researchers and it is one of the main arguments to support the role of the pig as an intermediate host in the generation of pandemic influenza viruses with avian HA genes (Kida et al., 1994, De Vleeschauwer et al., 2009a, Zhu et al., 2013). In our study, the replication rates of the original H9N2 and its virus distribution in the respiratory tract of pigs were similar to those described by De Vleeschauwer and colleagues in studies with a “purely avian” H5N2 LPAIV (De Vleeschauwer et al., 2009a). However, as described with other AIVs, infection with H9N2 did not result in efficient pig-to-pig transmission.

The serial pig passages induced a selective pressure on the virus that led to a broader tissue infection and increased virus replication after 4 passages. This consequence of the serial passaging was not only described by the increased binding of an avian H3N2 virus to Siaα2,6Gal receptors in pigs (Shichinohe et al., 2013), but also shown by the adaptation of H5N1 and H9N2 AIVs to ferrets (Sorrell et al., 2009, Herfst et al., 2012). It is interesting to mention that the broader tissue infection and the increased virus replication described after 4 passages were not correlated with the *in vitro* growth

kinetics of the virus. This finding confirms that *in vitro* results in continuous cell lines are often not relevant for the performance of the virus in the animal host. Similar findings were made in previous H9N2 studies, in which the pathogenicity and the virulence of the virus in pigs did not mimic the replication kinetics in MDCK and A549 cell lines (Qiao et al., 2012). In addition, our kinetic assay was performed with MDCK cells that are known to express in both avian-type (Siaα2,3Gal) and human-type (Siaα2,6Gal) cell receptors. Thus, this cell line is permissive to most influenza viruses and the differences due to a different receptor-binding affinity and not influence the cell line infection.

From pig passage 7 onwards replication of the H9N2 virus progressively decreased until it was lost. This loss-of-function may be explained by the negative effect of the selective pressure. It was already demonstrated that transmission of influenza viruses in ferrets incurs in a selective pressure. This pressure is supposed to generate a bottleneck that decreases viral diversity allowing minor variants to become dominant (Wilker et al., 2013). The stringency of those bottlenecks depends in part on the infection route (aerosol infection versus direct contact infection), and it can change during host adaptation (Varble et al., 2014, Moncla et al., 2014). In the case of H5N1 viruses and 1918 pandemic H1N1 it appeared that the selective pressure favoured the viruses with enhanced fitness (Wilker et al., 2013, Moncla et al., 2014). However, in a similar study done with the H7N9 subtype the bottleneck seemed to have the opposite effect and limited the transmission of the virus (Zaraket et al., 2015). Therefore, we hypothesized that the serial passages had a positive effect on the selection of virus variants with higher replication efficiencies. However, at a certain point the additive effect of successive selective pressures may become negative and this may result in a progressive decrease of the virus replication and excretion (Figure 1). As our serial passages were blind this progressive decrease in virus replication resulted in the inoculation of the subsequent animals with insufficient amounts of virus and a complete loss of the virus at passage 10. Extensive next generation sequencing (NGS) analysis of all passages would be needed to examine the genetic evolution of the virus during the 10 passages and to prove our hypothesis.

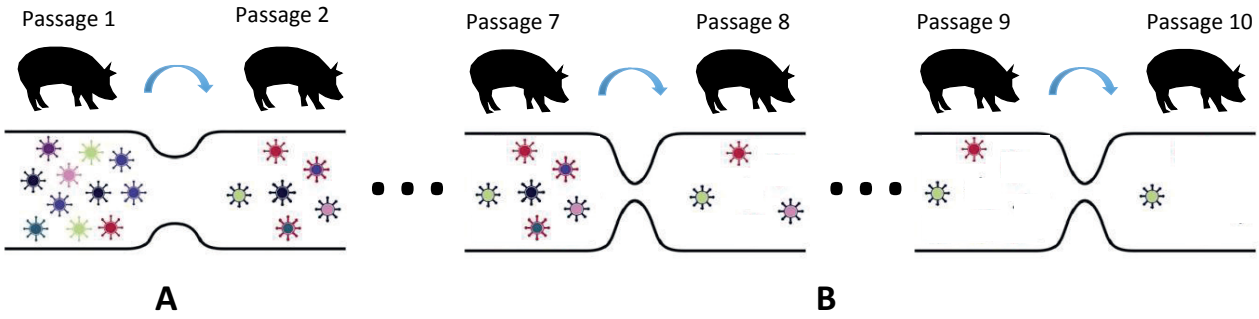

Figure 1. **(A)** Hypothesis on the effect of serial passaging in the generation of a selective pressure on the virus that selects for different virus variants but also decreases virus diversity. **(B)** The selective pressure comes more restrictive (narrower bottleneck) with more passages. This results in a decrease in virus fitness that will lead to the complete loss of the virus at passage 10 (Figure adapted from Moncla et al. 2016).

The fourth passage H9N2 virus showed enhanced direct-contact transmission between pigs when compared to the original virus. Though transmission of this virus did not result in high virus excretion by the direct-contact pigs, it was higher than that observed in previous H9N2 studies in pigs, or in other studies with H7N9 and other AIV subtypes (De Vleeschauwer et al., 2009b, The SJCEIRS H9 Working Group, 2013, Liu et al., 2014, Wang et al., 2016). However, transmission rates were lower when compared with H9N2 adaptation studies in mammals other than the pig. For example, after 15 passages with an avian H9N2 isolate in guinea pigs 3 out of 3 direct-contact guinea pigs excreted high amounts of virus (Sang et al. 2015). Of course it is difficult to compare our results in pigs with those obtained in other animal species because of the different animal models, the different virus strains and experimental conditions used. We selected swine because it is a natural influenza virus host that may act as an intermediary in the adaptation of AIVs to mammals, but also because pigs resemble humans in terms of physiology, anatomy and Sia receptor distribution. In contrast, other researchers used the ferret as it is considered the “gold standard” model for research on adaptation of avian influenza viruses to mammals (Belser et al., 2016). Guinea pigs or mice are also being used to evaluate transmission and/or virulence of “adapted” AIVs due to their easy management in experimental conditions. In addition to the evident physiological or physical differences posed by those animal models there are also differences in experimental design. In pigs, due the limitations posed by their size, we performed direct-contact transmission experiments. However, most

studies using ferrets or guinea pigs were more restrictive and considered efficient droplet transmission as a requirement for efficient adaptation (Sorrell. et al., 2009, Herfst et al., 2012, Imai et al., 2012, Sang et al., 2015). Although we consider that respiratory droplet transmission restricts virus transmission, it is clear that direct-contact transmission is one of the main pathways of influenza virus transmission in nature. We assume that a virus that transmits among pigs with a similar efficiency as endemic swine influenza viruses, will be efficiently transmitted in nature as well.

In conclusion, pigs are susceptible to H9N2 infection under experimental conditions and serial passages in pigs pose a selective pressure that enhances virus fitness. Thus, the pig may act as intermediate host for H9N2 AIVs. However, our results demonstrated that serial passages were not sufficient to confer efficient pig-to-pig transmission. This is consistent with the fact that H9N2 influenza viruses have recurrently infected pigs in nature but never became established in swine. Furthermore, previous experiments with H9N2 in ferrets also support these data. In those experiments reassortment with human seasonal H3N2 internal genes was necessary to achieve ferret-to-ferret transmission (Sorrel et al., 2009). However, introduction of 4 punctual mutations in the HA and 1 in the PB2 genes in a H5N1 virus was sufficient to achieve efficient droplet transmission in ferrets (Herfst et al., 2012). This reveals that adaptation of H9N2 AIVs to pigs is not a straightforward process and that a combination of molecular changes may be required to achieve full adaptation (of H9N2 AIVs in pigs).

The role of pH1N1 internal genes in the adaptation of avian H9N2 viruses to pigs

Our results demonstrate that the “pig-adapted” pH1N1-origin internal protein-genes may pose an advantage for the adaptation of H9N2 virus to pigs. This is consistent with previous studies in which similar reassortant H9N2 viruses showed enhanced transmission in pigs when compared with the original avian H9N2 (Qiao et al., 2012, Obadan et al., 2015). Although those viruses did not transmit as efficiently between pigs as the “swine-adapted” viruses, similar reassortant viruses showed efficient droplet transmission in ferrets (Kimble et al., 2011). These results also highlight the importance of the pH1N1 internal gene cassette in the adaptation of influenza A viruses to mammals. This “quadruple-reassortant” internal gene cassette contains

avian, human, North American and European swine-origin influenza virus genes and is widespread in swine and humans worldwide (Watson et al., 2015, Rajao et al., 2016). As mentioned in Chapter 4.4.3, the H9N2 reassortant virus used in our study was contaminated. Its internal genes were a combination of H9N2 and pH1N1-origin, instead of only pH1N1-origin. This contamination was possibly originated during the reverse genetic generation of the virus due to the unexpected presence of H9N2 internal genes plasmids. However, the serial passaging of these viruses clearly led to the selection of a virus possessing mainly pH1N1-origin internal genes, the only exception was the gene encoding the NP, which originated from the avian H9N2. The NP protein has been shown to play a role in the host adaptation by its interaction with the host-cell importin-α. This mediates the host-cell protein migration of the viral ribonucleoprotein to the nucleus and facilitates the evasion from the immune response in humans (Cauldwell et al., 2014). It was shown that human NP was necessary for efficient replication of H7 viruses in transgenic mice expressing human MxA, which is an interferon-stimulated protein that restricts virus replication (Deeg et al., 2017). Therefore, the fact that avian-origin NP remained in our virus and it was transmissible in swine contrasts with these experiments in transgenic mice. However, it raises the question how this “swine-adapted” virus would perform in humans as it was demonstrated that avian viruses require a human adapted NP to evade the negative effect of the MxA restriction factor in virus replication (Deeg et al., 2017).

Unlike the original H9N2, the reassortant H9N2 was not lost after serial passaging. Moreover, after 7 passages in pigs the reassortant virus showed enhanced transmission efficiency when compared with the original virus and with previous studies with H9N2:pH1N1 reassortant viruses in pigs (Qiao et al., 2012, Obadan et al., 2015), but also with the “adapted” H9N2 virus tested in Chapter 3. This higher transmission rate suggests that the combination of reassortment and serial passaging in pigs contributed to the adaptation of the reassortant H9N2 virus. This finding is important because both H9N2 and pH1N1 viruses are co-circulating in Asia and can infect pigs and humans increasing the possibilities of reassortment. The repeated introduction of such reassortant H9N2 viruses in humans or swine may result in the selection of virus variants with a transmission efficiency close to that of the mammalian adapted influenza viruses.

Genetic traits that influence the adaptation of avian H9N2 influenza viruses to pigs

Influenza A virus adaptation to a new host relies on 2 main mechanisms: aa mutations and genetic reassortment. Since the mid 1990s, few key point amino acids (*i.e.* aa 190, 225, 226 and 228 in the HA RBS or aa 627 and 701 in the PB2 protein genes) have been identified as important genetic markers for AIVs to achieve adaptation to mammals (Subbarao et al., 1993, Connor et al., 1994, Vines et al., 1998, Glaser et al., 2005). However, their role in the adaptation of AIVs to pigs remains uncertain.

For instance, the original H9N2 strain used in our experiments contained a leucine at position 226 of the HA RBS instead of glutamine that is typical for AIVs. This substitution, which was shown to increase the Siaα2,6Gal binding preference in human respiratory cells, has been also associated with higher replication rates and transmissibility of H3, H5, H7 and H9 subtype viruses in pigs and ferrets (Wan and Perez, 2007, Wan et al., 2008, Herfst et al., 2012, Van Poucke et al., 2013, Liu et al., 2014). Thus, the presence of this mutation in our original virus was supposed to be an adaptation advantage. However, in our experiments this substitution did not result in enhanced virus replication and tropism when compared to that of H5N2 AIVs, which did not have leucine, in pigs (De Vleeschauwer et al., 2009b). Thus, our data question the role of the HA-Q226L substitution in replication efficiency of H9N2 viruses in pigs. This is consistent with other studies, which show that residue 226 in HA RBS was not strictly determinative for the replication of H9N2 viruses in pigs or ferrets (The SJCEIRS H9 Working Group, 2013). Although comparative studies with HA-226L and HA-226Q would be required to evaluate the effect of the HA-Q226L substitution in pigs, our results show that the adaptation process of H9N2 viruses to pigs is much more complex than the acquisition of a single point mutation in the HA RBS.

During the pig passages with the wholly avian H9N2 virus, we observed a new aa substitution, which was related with a broader tissue tropism and increased transmission. In addition, we demonstrated that upper and lower respiratory tract selected for different virus variants. Similar findings were made in ferrets (Lakdawala et al. 2015) and they may be due to the different receptor distribution present in the lungs compared to the upper respiratory tract (Van Poucke et al., 2010, Trebbien et al., 2011). During the serial passages with the reassortant virus (Chapter 4), we also observed the emergence of new substitutions. Only 1 out of the 19 mutations

described in both experiment, the HA-D225G substitution, was common to both experiments. This substitution is located in the HA receptor binding site and is known to result in a difference in the HA RBS surface structure, see Figure 2. Furthermore, the interaction of the aa on the RBS with the Sia receptor is dependent on electric charges and this mutation results in the substitution of a negative charged (aspartic acid) aa by an uncharged one (glycine). Thus, it may affect the interaction of the virus with Sia. However, as demonstrated in Chapter 3, this mutation was maintained when the virus was progressively getting lost during the serial passages. Interestingly, all 47 swine and 12 human H9N2 field isolates available in GenBank also showed this HA-D225G mutation, while HA-Q226L was lacking in some of them (see Table 3).

Table 1. Number of swine and human H9N2 field isolates available in GenBank that contain the HA-D225G and /or the HA-Q226L substitutions.

H9N2 virus isolated from	**Number of isolates with … substitution**	
	D225G	**Q226L**
Humans (n=17)	17/17 (100%)	14/17 (82%)
Pigs (n=42)	42/42(100%)	25/42 (60%)

The HA-D225G substitution has been previously described in the literature as an important host specificity marker in H1 viruses (Taubenberger and Kash, 2010, Romero-Tejeda and Capua, 2013, Richard et al., 2014). In 1918 and 2009 H1N1 pandemic viruses, this substitution has been associated with increased Siaα2,3Gal tropism conferring those viruses dual Siaα2,6Gal and Siaα2,3Gal receptor binding affinity (Tumpey et al., 2007, Belser et al., 2011, Lakdawala et al., 2015). In pigs and in humans Siaα2,3Gal receptors are predominantly found in the lungs (Van Poucke et al., 2010). The emergence of this mutation could therefore explain the enhanced virus replication in the lungs. However, *in vitro* sialylglycoprotein binding assays H9N2 viruses containing HA-225G and HA-226L, showed only slightly increased Siaα2,6Gal binding affinity (Matrosovich et al., 2001, Lakdawala et al., 2015). In addition, this substitution has never been described as an *in vivo* adaptation marker of other AIV subtypes such as H5, H7 or H9 (Sorrell et al., 2009, Herfst et al., 2012, Liu et al, 2014). Though our data suggest that the HA-D225G substitution is an important marker for

adaptation of H9N2 viruses to pigs, further lectin-binding analysis will be needed to confirm the importance of this mutation for the receptor-binding affinity.

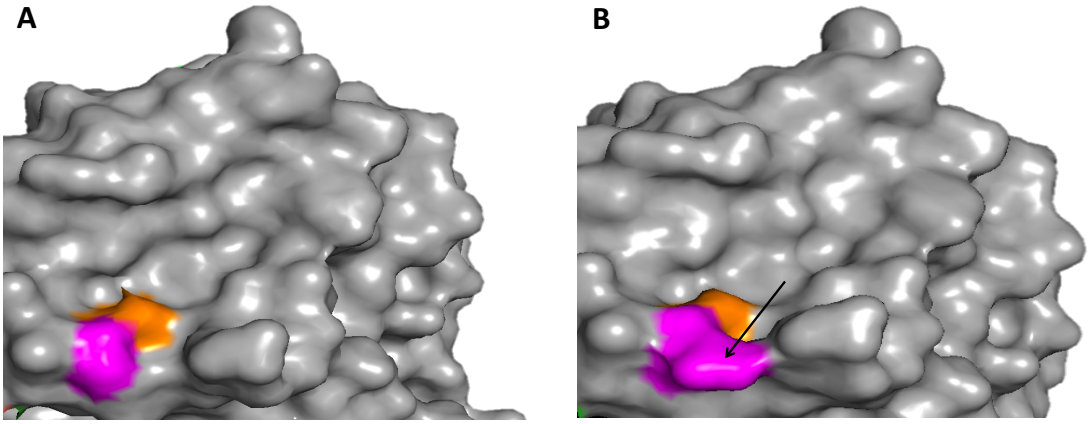

Figure 2. Comparison of the surface representation of the receptor-binding site in the HA1 monotrimer of: (A) A/quail/Hong Kong/G1/1997 (H9N2) virus before serial passaging containing D225 and L226, and (B) the same virus after 4 passages in pigs containing G225 and L226. In both figures aa 225 and 226 are highlighted in magenta and orange respectively. The arrow points to the different 3d representation produced by the substitution in the aa 225.

The second mechanism of adaptation is the reassortment of gene segments between 2 (or more) different influenza viruses that co-infect the same cell. This mechanism is more drastic but genetic reassortment involving AIVs has been involved in the generation of most endemic influenza viruses in pigs and humans. Actually, the H3N2 and H1N2 SIV that are endemic in European pigs and the H1N1, H3N2 and H1N2 SIV that circulated in North America between 1998 and 2009 were generated by reassortment of avian, and human viruses. In humans, H2N2 and H3N2 pandemic viruses were also reassortants containing avian-origin surface genes combined with internal genes of human-adapted viruses. Furthermore, the 2009 pandemic H1N1 virus was a reassortant containing avian, human and swine-origin genes. Its particular internal gene cassette became efficiently adapted to pigs and humans and has been described to enhance human transmission of other AIVs such as H5, and/or H9 (Mehle and Doudna, 2009, Kimble et al., 2011, Lakdawala et al., 2011, Sun et al., 2011, Imai et al., 2012, Zhang et al., 2012, Abente et al., 2016). Our experiments confirmed previous reports and, besides the NP protein-gene, we demonstrated that pH1N1 internal genes

were selected upon adaptation to pigs. After 7 passages the reassortant virus was transmitted at higher rates to 4 our out of 6 contact-pigs. Those transmission rates were higher when compared to other AIVs but were still not at the level of pH1N1, which transmitted to 6 out of 6 contact-pigs. To elucidate the reason why the reassortant H9N2 virus was not transmitted to all the direct-contact pigs, genetic analysis of the viruses isolated from the transmission experiments should be performed. In addition, a subsequent transmission experiment performed with the virus excreted from the direct-contact pigs may result in transmission to all direct-contact animals. This would be in agreement with the H5N1 adaptation experiment performed by Herfst and colleagues in which they obtained efficient ferret-to-ferret transmission after 10 serial passages and 3 transmission experiments (Herfst et al., 2012)

Conclusions and implications

- Serial passages of an avian H9N2 influenza virus in pigs resulted in an enhanced virus replication and transmission. However, it was less efficient when compared to the endemic swine influenza viruses. This is consistent with the natural scenario in which avian H9N2 viruses are recurrently isolated from pigs but never became established in swine populations. Similarly, in experimental studies in ferrets efficient droplet transmission was only obtained upon reassortment with mammalian adapted strains.
- The combination of pH1N1 internal genes and serial pig passages enhanced H9N2 virus replication in and transmission between pigs.
- The HA-D225G substitution emerged in both experimental set-ups. It is present in the RBS of the HA. Thus, it that may have an important role in the adaptation of avian H9N2 to pigs. Nevertheless, further research will be needed to elucidate the exact role of this mutation.
- Our studies put light in the adaptation of H9N2 to pigs, which is an interesting model to evaluate AIVs transmission to mammals. However, up to nowadays the pH1N1 was the only swine-origin virus that became established in humans. So far, the ferret is the reference animal model to evaluate the pandemic threat of influenza viruses. It could be interesting to perform further

transmission experiments in ferrets with the viruses generated in our studies. On the other hand, neither pigs nor ferrets are humans and ferrets seem to be more susceptible to influenza viruses than any other mammalian species.

- To conclude, our studies showed that adaptation of H9N2 viruses to pigs is a complex process that involves mutations in single protein-genes as well as the interactions between those genes.

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6 SUMMARY - SAMENVATTING

Summary

Avian H9N2 influenza viruses with enhanced human receptor-binding affinity have been recurrently isolated from pigs and humans since 1998. This isolation increased their public health concern. In 2009, the emergence of the 2009 pandemic H1N1 virus in swine put the scope on swine as host where novel pandemic viruses could be generated. The hypothesis that swine may play a role as intermediate hosts and/or "mixing vessels" in which AIVs may adapt to mammals increasing their potential threat to generate novel pandemics was enunciated more than 20 years ago. However, it has never been proved and there are arguments for and against it.

In **Chapter 1**, a general introduction to influenza A virus taxonomy, structure, replication cycle and virus evolution is given. In the second section the epidemiology of avian, human and swine influenza viruses is summarized. The third and the fourth sections depict the molecular mechanisms that drive the host range restriction and the avian viruses with higher pandemic potential. Later, the fifth and sixth sections present a summary of the hypothesized role of the pig in the adaptation of AIVs to mammals and the use of the pig as research model.

Chapter 2 outlines the aims of the thesis. The first aim was to adapt an entire H9N2 AIV to pigs and to contribute to the general understanding of the mechanisms of adaptation of the AIVs to mammals. Moreover, our second aim was to evaluate the impact of reassorting the pH1N1 backbone with H9N2 AIV on replication capacity and transmission in pigs.

Chapter 3 deals with the adaptation of an entire avian H9N2 virus to pigs by serial bind passages. In this chapter, we characterized virus replication in the entire respiratory tract during all passages and we selected the best replicating virus to perform a direct contact transmission experiment to evaluate if the better performance during the passages is also correlated with enhanced transmissibility. We also compared genetically the original material with the virus selected for the transmission experiment. The original H9N2 virus caused a productive infection in pigs with a predominant tropism for the nasal mucosa. In contrast, after 4 passages the virus

replicated in the entire respiratory tract. Subsequent passages were associated with reduced virus replication in the lungs and infectious virus was lost at passage 10. The broader tissue tropism after 4 passages was associated with an amino acid residue substitution at position 225, within the receptor-binding site of the hemagglutinin. In the direct contact transmission experiment, the passage 4 virus showed enhanced transmission when compared to the original virus. Nevertheless, it was significantly lower when compared to that of the pH1N1. Our data demonstrate that serial passaging of H9N2 virus in pigs enhances its replication and transmissibility. However, full adaptation of an avian H9N2 virus to pigs likely requires a more drastic approach.

In **Chapter 4** the effect of the reassortment with pH1N1 internal genes combined with the serial passaging in pigs was examined. In this chapter, we repeated the experimental set up described in Chapter 3 but the original avian virus was substituted by a reassortant virus containing H9N2 HA and NA protein-genes in the pH1N1 backbone. The aim of this experiment was to evaluate if the introduction of pH1N1 internal genes offered an advantage for adaptation of H9N2 AIVs in pigs. The viral replication was increased in contrast to that of the original avian H9N2 virus after serial passages. Furthermore, it did not lose its replication capacity after 10 passages in pigs in comparison to the original avian H9N2 virus. We selected the virus after 7 passages to perform the direct-contact transmission experiment. This virus was excreted by 4 out of 6 animals at similar rates when compared to pH1N1. However, the genetic analysis revealed that the original virus was contaminated and the internal genes present in the original stock contained a mixture of H9N2 and pH1N1 internal genes. Interestingly the serial passaging selected for pH1N1 internal genes and in passage 7 virus only NP-coding gene was completely of H9N2-origin. The passage 7 virus also contained the HA-D225G substitution described in Chapter 3. Our data demonstrate that serial passaging of a reassortant H9N2 virus in pigs selected the same mutation as the one selected by the same experimental set-up performed with the wholly avian virus. However, it shows that adaptation to pigs is a complex process that may require the combination of reassortment with point mutations.

In **Chapter 5,** the experimental studies are discussed. In general, our studies demonstrated that the adaptation of AIVs to pigs is a very complex process that needs drastic genetic changes to be achieved. Adaptation of avian H9N2 to mammals can be achieved by the combination of reassortment with efficiently adapted internal genes and serial passages. However, it remains questionable whether the results described are extrapolatable to other strains or subtypes and to the possible adaptation to humans. By setting-up that experimental model we have generated the basis to generate better adapted virus and genetically characterize them in order to put light in the understanding of the adaptation process of AIVs.

Samenvatting

Aviaire H9N2-influenzavirussen met verhoogde bindingsaffiniteit voor de menselijke receptor werden sinds 1998 herhaaldelijk geïsoleerd uit varkens en mensen. Hun vondst brengt een verhoogde risico voor de volksgezondheid met zich mee. In 2009 benadrukte de opkomst van het pandemisch H1N1-virus uit 2009 (pH1N1) bij varkens het belang van varkens als gastheer waarin nieuwe pandemische virussen zouden kunnen ontstaan. De hypothese dat varkens een rol kunnen spelen als tussengastheren en/of "mengvaten" waarin aviaire influenzavirussen (AIV's) zich kunnen aanpassen aan zoogdieren en zo een verhoogde potentiële bedreiging vormen voor het ontstaan van nieuwe pandemieën, werd meer dan twintig jaar geleden geformuleerd. Dit werd echter nooit bewezen en er zijn argumenten voor en tegen deze hypothese.

In **hoofdstuk 1** wordt een algemene introductie gegeven over de taxonomie, structuur, replicatiecyclus en virusontwikkeling van influenza A-virussen. In de tweede sectie wordt de epidemiologie van aviaire, humane en varkensinfluenzavirussen samengevat. De derde en de vierde sectie duiden de moleculaire mechanismen die het gastheerbereik beperken en beschrijven de aviaire virussen met een hoger pandemisch potentieel. Vervolgens wordt in de vijfde en zesde sectie een samenvatting gegeven van de veronderstelde rol van het varken in de aanpassing van AIV's aan zoogdieren en van het gebruik van varkens als model voor het onderzoek naar deze adaptatie.

Hoofdstuk 2 schetst de doelstellingen van het proefschrift. Het hoofddoel was om een H9N2 AIV aan te passen aan varkens en om bij te dragen aan het algemene begrip van de mechanismen van aanpassing van de AIV's aan zoogdieren.

Hoofdstuk 3 behandelt de aanpassing van een volledig aviair H9N2-virus aan varkens door opeenvolgende blinde passages. In dit hoofdstuk hebben we de virusreplicatie in het hele respiratoire kanaal gekarakteriseerd tijdens alle passages en hebben we het best aangepaste virus geselecteerd voor het uitvoeren van een direct-contact transmissie-experiment om te evalueren of de betere prestaties tijdens de passages

ook gecorreleerd zijn met verhoogde overdraagbaarheid. We hebben ook genetisch het startmateriaal vergeleken met het virus dat geselecteerd werd voor het transmissie-experiment. Het oorspronkelijke H9N2-virus veroorzaakte een productieve infectie bij varkens met een overheersend tropisme voor de neusslijmvliezen. Na vier passages repliceerde het virus daarentegen in het hele respiratoire kanaal. Volgende passages werden geassocieerd met gereduceerde virusreplicatie in de longen en het besmettelijke virus ging verloren bij passage tien. Het uitgebreider weefseltropisme na vier passages werd geassocieerd met een aminozuursubstitutie op positie 225 binnen de receptorbindingsplaats op het hemagglutinine. In het direct-contact transmissie-experiment vertoonde het virus van passage vier verbeterde transmissie in vergelijking met het oorspronkelijke virus. Desondanks was uitscheiding significant lager in vergelijking met die van pH1N1. Onze gegevens tonen aan dat seriële passages van H9N2-virus bij varkens de replicatie en overdraagbaarheid verbetert. Echter, volledige aanpassing van een aviair H9N2-virus aan varkens vereist waarschijnlijk een drastischere aanpak.

In **hoofdstuk 4** werd het effect van reassortering van de aviaire influenza H9N2 met interne genen van pH1N1 op de replicatiecapaciteit in varkens onderzocht door middel van seriële passages. In dit hoofdstuk herhaalden we de proefopzet beschreven in hoofdstuk 3, maar het oorspronkelijke virus werd vervangen door een reassortant virus dat de HA- en NA-eiwitgenen van het H9N2-virus en de interne genen van het pH1N1-virus bevat. De bedoeling van dit experiment was om te evalueren of de introductie van pH1N1 interne genen een voordeel betekende voor de aanpassing van H9N2 AIV's aan varkens. Dit virus presteerde beter dan het oorspronkelijke virus na tien passages werd nog steeds infectieus virus gedetecteerd. We hebben het virus na zeven passages geselecteerd om een direct-contact transmissie experiment uit te voeren. Dit virus werd uitgescheiden door vier van de zes dieren aan hoeveelheden vergelijkbaar met deze voor pH1N1. Echter, uit de genetische analyse bleek dat het oorspronkelijke virus gecontamineerd was en dat de interne genen aanwezig in de oorspronkelijke stock een mengsel van H9N2 en pH1N1 interne genen bevatte. Interessant is dat de seriële passages selecteerden voor pH1N1 interne genen en in het virus van passage zeven was enkel het NP-coderend gen volledig van H9N2-oorsprong.

Het virus van passage zeven bevat ook de substitutie HA-D225G beschreven in hoofdstuk 3. Onze gegevens tonen aan dat opeenvolgende passages van een reassortant H9N2-virus bij varkens dezelfde mutatie geselecteerd heeft als die geselecteerd door dezelfde experimentele opstelling uitgevoerd met het volledig aviaire virus. Het blijkt echter dat aanpassing aan varkens een complex proces is dat mogelijk de combinatie van reassortering met puntmutaties vereist.

In **hoofdstuk 5** worden de experimentele studies besproken. Over het algemeen hebben onze studies aangetoond dat de aanpassing van AIV's aan varkens een zeer complex proces is dat drastische genetische veranderingen vereist. Aanpassing van aviair H9N2 aan zoogdieren kan worden bereikt door de combinatie van reassortering met efficiënt aangepaste interne genen en seriële passages. Echter, het blijft onzeker of de resultaten die hier beschreven worden geëxtrapoleerd kunnen worden naar andere stammen of subtypes en naar de mogelijke adaptatie aan mensen. Door het experimentele model op te zetten, hebben we de basis gelegd om beter aangepaste virussen te genereren en om deze virussen genetisch te karakteriseren, met als doel licht te werpen op het begrijpen van het adaptatieproces van AIV’s.

7 CURRICULUM VITAE

Personalia

Jose Carlos Mancera Gracia was born in Zaragoza, Spain on January 11 1986. In 2004 he completed his secondary education at the Instituto Pablo Gargallo in Zaragoza and started the study of veterinary medicine at the Faculty of Veterinary Medicine, Zaragoza University, from which he graduated in 2011. In August 2011 he was granted with a Leonardo da Vinci scholarship and he started an internship at the Nutreco B.V. Swine Research Centre in Boxmeer (the Netherlands). In September 2012 he joined the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University where he studied the possibilities of adaptation of avian H9N2 influenza viruses to swine. His PhD studies were funded by the European Commission FLUPIG FP7 project (FP7-GA 258084).

Publications

Publications in international scientific peer-reviewed journals

Mancera Gracia, J. C., Van den Hoecke, S., Richt, J. A., Ma, W., Saelens, X. and Van Reeth, K. (2017). A reassortant H9N2 influenza virus containing 2009 pandemic H1N1 internal-protein genes acquired enhanced pig-to-pig transmission after serial passages in swine. *Scientific reports, 7, 1323.*

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