Aus dem Bereich Biologie, der Medizischen Fakultät der
Universität des Saarlandes, Homburg/Saar und dem
Institut für Immunologie/Centre de Recherche Public de la Santé,
Luxembourg

Molekulare Charakterisierung von Influenza A Viren - Epidemiologie und Genetik

Molecular characterization of influenza A viruses - epidemiology and genetics

DISSERTATION

der Medizinischen Fakultät

der UNIVERSITÄT DES SAARLANDES

zur Erlangung des Grades eines Doktors der Naturwissenschaften

2010

vorgelegt von

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geb. am 22.06.1981 in Perleberg

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List of abbreviations

A	adenine
aa	amino acid
bp	base pair
BSA	bovine serum albumin
BLAST	basic local alignment search tool
c	complementary
C	Celsius or cytosine
CDC	Centers for Disease Control and Prevention
CGM	cell growth medium
CO_2	carbon dioxide
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dNTP	deoxyribonucleotidetriphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMEM	essential modified Eagle's medium
FBS-HI	heat inactivated fetal bovine serum
G	guanine
H/HA	hemagglutinin
HCl	hydrogenic chloric acid
HEPES	hydroxyethyl-piperazin-ethanesulfonic acid
HI	hemagglutinin inhibition
HPAI	highly pathogenic avian influenza
Ig	immunoglobulin
IFN	interferon
IL	interleukin
ILI	Influenza-like illness

kb	kilobase
KCl	potassium chloride
LB	Luria broth base
LPAI	low pathogenic avian influenza
MDCK	Madin-Darby Canine Kidney
$MgCl_2$	magnesium chloride
M	matrix protein
mRNA	messenger RNA
N/NA	neuraminidase
NAI	neuraminidase inhibitor
NP	nucleoprotein
NS/NEP	nonstructural/nuclear export protein
OD	optical density
OIE	World Organisation for Animal Health
PA	polymerase acid protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PB1 & PB2	polymerase basic protein 1 & 2
RNA	ribonucleic acid
RT	reverse transcription
SAα2,3Gal	sialic acid α2,3 linked galactose
SIV	swine influenza virus
SwC	swine contact
T	thymine
TCID	tissue culture infectious dose
TE	Tris-EDTA buffer
TLR	Toll-like receptor
TNF	tumor necrosis factor
UV	ultraviolet
VGM	virus growth medium
VN	virus neutralization
WHO	World Health Organization

Single amino acid code

single letter code	amino acid name	abbreviation
A	Alanine	Ala
R	Arginine	Arg
N	Asparagine	Asn
D	Aspartic acid	Asp
C	Cysteine	Cys
E	Glutamic acid	Glu
Q	Glutamine	Gln
G	Glycine	Gly
H	Histidine	His
I	Isoleucine	Ile
L	Leucine	Leu
K	Lysine	Lys
M	Methionine	Met
F	Phenylalanine	Phe
P	Proline	Pro
S	Serine	Ser
T	Threonine	Thr
W	Tryptophan	Trp
Y	Tyrosine	Tyr
V	Valine	Val

L-amino acids are displayed with capital letters using the one-letter-code or the three-letter-code.

Zusammenfassung

Influenza A Viren können aufgrund der Zusammensetzung der Oberflächenmoleküle in zahlreiche Virussubtypen unterteilt werden, abhängig von 16 Varianten der Hämagglutinin (H) und 9 der Neuraminidase (N) Gene. Fast alle Kombinationen wurden bisher in Vögeln gefunden. Im Menschen kommen hauptsächlich die Subtypen H1N1 und H3N2 vor und rufen jährlich Epidemien und seltener Pandemien hervor. Die Evolution der Influenzaviren wird durch zwei einzigartige Mechanismen vorangetrieben, welche im Fokus dieser Arbeit stehen. Zum Einen dem Austausch von Gensegmenten ermöglicht durch das segmentierte Virusgenom - „*antigenic shift*“, und zum Anderen durch die kontinuierlich ändernden Oberflächenproteine - „*antigenic drift*“.

Eine grundlegende Veränderung an der Spaltungsstelle des Hämagglutininproteins geht einher mit signifikanter Morbidität und Mortalität in Geflügel und wird hochpathogene aviäre Influenza (HPAI) genannt. Nachdem 2006, HPAI Viren des Subtyps H5N1 zum ersten Mal in Afrika gefunden wurden, wurden ein Jahr später neue Ausbrüche gemeldet. Unsere Studie zeigte in phylogenetischen Analysen, dass die Genome dieser HPAI Viren (H5N1) näher mit Stämmen aus Nigeria aus 2006 verwandt waren, als mit Stämmen, die außerhalb des Landes identifiziert wurden. Die Genome in sechs von acht Viren waren das Ergebnis von mindestens drei separaten Neu-Sortierungen von Gensegmenten zwischen den vorher gefundenen Viren der Sublinien A und C. Weiterhin zeigten unsere Ergebnisse, dass HPAI (H5N1) Viren trotz extensiver Ausrottungskampagnen ungebremst in Nigeria zirkulierten, welches weit verbreitete Neu-Sortierungen zwischen genetisch unterschiedlichen Linien zuließ. Interessanterweise stammten in allen neusortierten Viren die Nichtstrukturproteine von der Sublinie C und enthielten gleichzeitig zwei charakteristische Aminosäuren im Unterschied zu der Sublinie A. Die hohe Prävalenz von neusortierten Viren im Jahr 2007 und die gleichzeitige Abwesenheit von denselben Viren außerhalb dieser Region, deuteten darauf hin, dass die Wiedereinführung von H5N1 Viren von Afrika nach Eurasien eher selten auftrat.

Humane Influenzaviren gelten als eine der wichtigsten Pathogene, die respiratorische Erkrankungen im Menschen verursachen. Trotz effizienter Behandlungsmöglichkeiten gegen die Virusgrippe bleibt es von zentraler Bedeutung die Wirksamkeit antiviraler Medikamente zu testen. Im Winter 2007/2008 traten neue Virusvarianten des Subtyps

H1N1 auf. Diese Viren besaßen eine Genmutation, welche Resistenz gegen den Neuraminidaseinhibitor Oseltamivir verleiht. Durch ihre schnelle Verbreitung wurden diese Viren zur vorherrschenden Variante saisonaler Grippeviren H1N1. In unserer Studie verglichen wir epidemiologische und klinische Daten von Patienten, infiziert mit Oseltamivir-resistenten oder -sensitiven Viren und zeigten, dass die Fälle resistenter Influenza H1N1 in Luxemburg nicht mit vorheriger Behandlung oder Prophylaxe mit Oseltamivir gekoppelt waren. Nach anfänglich lokalem Auftreten, breiteten sich die resistenten Stämme parallel zu den sensitiven Stämmen aus und beide Virusvarianten zeigten eine ähnliche Epidemiologie und klinische Symptome. Phylogenetische Analysen zeigten, dass Oseltamivir-resistente von -sensitiven Viren unterscheidbar waren, aufgrund spezifischer genomischer Marker in zwei Gensegmenten. Spezifische Mutationen, die in Proteinen von resistenten Varianten auftraten, fehlten in sensitiven Viren, was möglicherweise darauf hindeutet, dass resistente Virenvarianten mit Mutationen eine erhöhte Fitness aufwiesen verglichen zu resistenten Viren ohne diese zusätzlichen Mutationen.

Nach dem Auftreten eines neuen humanen Influenzavirus im Jahr 2009 wurde es bedeutend den Antikörpergehalt von menschlichen Seren zu messen um einen Schutz gegen das neue Virus abzuschätzen. Serologische Daten über Schweininfluenzaviren (SIV) von Menschen mit beruflichem Kontakt zu Schweinen liegen bisher nur für die USA vor. In der hier beschriebenen Studie aus Luxemburg wurden neutralisierende Antikörper gegen das pandemische Influenzavirus H1N1 von 2009, sowie gegen das aviär-ähnliche enzootische H1N1 SIV in Schweinkontakten mit einer Kontrollgruppe verglichen. Wir zeigten, dass professionelle Schweinearbeiter in Westeuropa eine höhere Konzentration neutralisierender Antikörpern gegen beide getesteten Viren besaßen als die Kontrollpersonen. Ein geringer Anteil der Seren der Kontrollgruppe zeigte einen neutralisierenden Effekt gegen eines oder beide Viren, obwohl vorheriger Kontakt zu Schweinen unwahrscheinlich war. Die Ergebnisse deuten darauf hin, dass sequenzielle Infektionen mit humanen saisonalen Influenzaviren des Subtyps H1N1 möglicherweise die Chancen auf serologische Kreuzreaktionen mit antigenetisch unterschiedlichen H1N1 Viren erhöhen. Es ist anzunehmen, dass diese kreuzreaktiven Antikörper einen gewissen Grad an Schutz vor Infektionen mit neuen Virusvarianten bieten. Die hier vorliegende

Arbeit verbessert das Verständnis der Influenza A Viren im Bereich molekularer Epidemiologie und komplexer Mechanismen der Virusevolution.

Abstract

Influenza A viruses are distinguished into various subtypes based on the composition of their surface proteins with 16 different variants of hemagglutinin (H) and 9 different variants of neuraminidase (N) genes. Almost all combinations were identified in avian species. In humans, only subtypes H1N1 and H3N2 are present, causing annual epidemics and rarely pandemics. Due to the segmented genome, two unique mechanisms thrive the evolution of influenza A viruses - antigenic shift or the exchange of gene segments or reassortments and antigenic drift, the continuous change of antigenicity of the surface proteins, which will be the focus of this work.

A major change in the cleavage site of the hemagglutinin (HA) protein results in huge morbidity and mortality in infected poultry, named highly pathogenic avian influenza (HPAI). After initial introduction of these viruses in Africa, in 2006 HPAI spread from Nigeria to neighboring countries. In the first study, complete genome sequencing of HPAI (H5N1) viruses from 2007 and subsequent phylogenetic analysis revealed that all gene sequences were more closely related to the first strains of sublineage A and C found in Nigeria in 2006 than to any strain found outside of the country. Six out of eight viruses had evolved by at least three reassortment events from previously identified sublineages A and C viruses. Our results suggested that HPAI (H5N1) viruses initially imported into Nigeria in 2006 have been gradually replaced by various reassortments. Interestingly, in all reassortants nonstructuralprotein genes were derived from sublineage C with two characteristic amino acids (compared to sublineage A). If the high prevalence of reassortants was typical for West-Africa in 2007, the absence of such reassortments anywhere else suggests that reintroductions of H5N1 from Africa into Eurasia must be a rare event. Further, the results indicated that despite extensive eradication campaigns HPAI (H5N1) continued to circulate in Nigeria, allowing various reassortment events between viruses from diverse genetic lineages.

Human influenza viruses are one of the major pathogens worldwide causing respiratory diseases with significant morbidity and mortality in risk groups. Effective treatment against influenza viruses is available; however, assessment of viral drug susceptibility remains important. During the season 2007-2008, new H1N1 virus variants emerged containing a mutation in their neuraminidase (NA) gene, which conferred resistance to the

neuraminidase inhibitor oseltamivir. These viruses spread efficiently and eventually became the prevailing variant of H1N1 viruses. To study if their emergence was associated with treatment with oseltamivir, we investigated clinical and epidemiological data of patients infected with oseltamivir-resistant in comparison to drug-sensitive viruses. Human cases of oseltamivir-resistant influenza A H1N1 emerging in 2007-2008 in Luxembourg were not associated with treatment, prophylaxis or stockpiling of oseltamivir. Following initial local seeding, resistant strains spread synchronously to sensitive strains causing a similar epidemiology and clinical symptoms. However, phylogenetic analysis revealed genomic markers segregating oseltamivir-resistant from oseltamivir-sensitive viruses in NA and polymerase basic protein 2 (PB2) gene sequences. Specific mutations were present in resistant viruses but absent in drug-sensitive variants indicating that drug-resistant variants that contained these mutations most probably resulted in enhanced fitness compared to resistant virus variants without these additional mutations.

Finally, with emergence of a new influenza A virus of swine-origin in 2009, serological studies to assess antibody protection levels in humans became of interest. Serological studies on swine influenza viruses (SIVs) in humans with occupational exposure to pigs have only been reported from the Americas, but not from Europe. Thus in this study, we analyzed neutralizing antibodies against the pandemic H1N1 2009 influenza virus and an avian-like, enzootic H1N1 SIV in swine contacts in Luxembourg compared to a matched general population. We showed that professional swine contacts in Western Europe elicited more frequently neutralizing antibodies against both H1N1 viruses than controls. Part of the general population, however, also tested positive against either one or both viruses, while exposure to them was unlikely. Sequential infections with variants of human seasonal H1N1 viruses may have increased the chance of serological cross-reaction with the antigenically distinct H1N1 viruses tested for, and we assume that these cross-reactive antibodies may provide some level of cross-protection. Further studies are required to determine to what extent the serological responses correlate with infection.
In conclusion, this work advances the knowledge about influenza A virus' molecular epidemiology and complex mechanisms of virus evolution.

Chapter I: Introduction

1. Historical background

The history of influenza epidemics and pandemics can be traced back for the past three hundred years. The term influenza originated from the Latin "*influentia*" referencing the influence of the stars or astrology on illness. It appeared first in 1729, borrowed from the Italian *influenza* "influenza, epidemic" but originally derived from the Latin "visitation, influence (of the stars)," and naming the first agreed influenza pandemic in humans. The term influenza was often applied since the mid-19th century to refer to severe colds and its shortened form "flu" became used since 1839 (Harper 2001; Potter 2001).

In 1901, the virus responsible for highly pathogenic avian influenza (HPAI) or 'fowl plague' was the second (after foot and mouth disease virus) to be identified as an ultra-filterable agent (i.e. able to pass through ceramic filters that removed bacteria and yeasts) (Centanni 1901; Lode 1901). It was not until 1934 that Burnet and Ferry described the use of embryonated fowls' eggs to propagate 'fowl plague' virus (Burnet 1934). Nonetheless, only in 1955 could the 'fowl plague' be associated to influenza viruses (Wright 2007).

The first human influenza virus isolation was successfully carried out in 1933 (Smith 1933). Since then, the history of infection could be recorded, and advancement in laboratory diagnosis allowed its confirmation by experimental assays. Today's monitoring of human influenza virus circulation and variation lies with the World Health Organization (WHO), who has established a tight, worldwide network of more than a hundred research laboratories and acts as a key player in anticipating epidemics (WHO 2010c).

At present, influenza viruses are one of the most studied viruses, and the most studied respiratory virus at all. A query for the keyword 'influenza' in the largest online biomedical library, the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov), returned over 55'000 scientific publications on May 11, 2010. As

older publications or books addressing the topic of influenza are unlikely to be included, this figure is only an indicator of recent progress. In addition, the significance of numerous influenza studies cannot be assessed without knowing the impact of the disease on public and animal health. Influenza viruses were responsible for several pandemics with devastating consequences in the past, and today, annual epidemics of seasonal influenza, still claim 250'000 to 500'000 deaths (Nicholson 2003). Thus, influenza remains one of the major respiratory agents worldwide causing high levels of morbidity and mortality, despite effective vaccines and potent antivirals. Noteworthy, various outbreaks of HPAI viruses in poultry flocks resulted in eradication campaigns culling millions of birds and were paired with high economic losses (Wright 2007).

2. The virus

2.1. Classification

Influenza viruses belong to the family *Orthomyxoviridae*, which includes the genera *Influenzavirus A; B; C*; *Thogotovirus* and *Isavirus*. All members of this family possess negative-sense; single stranded, and segmented RNA genome. The focus of this work are influenza A viruses classified into subtypes based on the antigenicity of their hemagglutinin (HA) and neuraminidase (NA) proteins. The strain nomenclature describes the genus (type) of virus, the host (omitted if human), the geographic origin, the strain number, and the year of detection, followed by their subtype (*e.g.* A/chicken/Nigeria/OG2/2007 [H5N1]). As of today, 16 HA subtypes (H1-H16) and 9 NA (N1-N9) subtypes for influenza A viruses have been identified (Wright 2007).

2.2. Structural and genomic organization

Influenza A viruses are enveloped, roughly spherical viruses with a size of 80-120 nm in diameter containing segmented RNA molecules, which correspond to eight genes encoding 11 proteins (Figure 1 A). Figure 2 presents a model of the overall structure of the influenza A virus. The viral envelope is derived from the phospholipid membrane of the host cell and is spiked with HA, NA and M2 proteins, whereas the M1 protein is located below the viral envelope (Ruigrok 1998). The HA is a trimer, consisting of three

individual HA monomers, while NA is a tetramer (Varghese 1983; Colman 1998; Steinhauer 1998). The four times more abundant HA envelope protein is synthesized in the infected cell as a single polypeptide chain (HA0) with a length of about 560 amino acid (aa) residues, which is subsequently cleaved into two subunits HA1 and HA2 (Steinhauer 1998; Wright 2007). These subunits remain covalently linked to each other through disulphide bonds. Cleavage of HA0 is essential for the molecule to be able to mediate membrane fusion between the viral envelope and the host cell membrane, as discussed in Section 2.5.

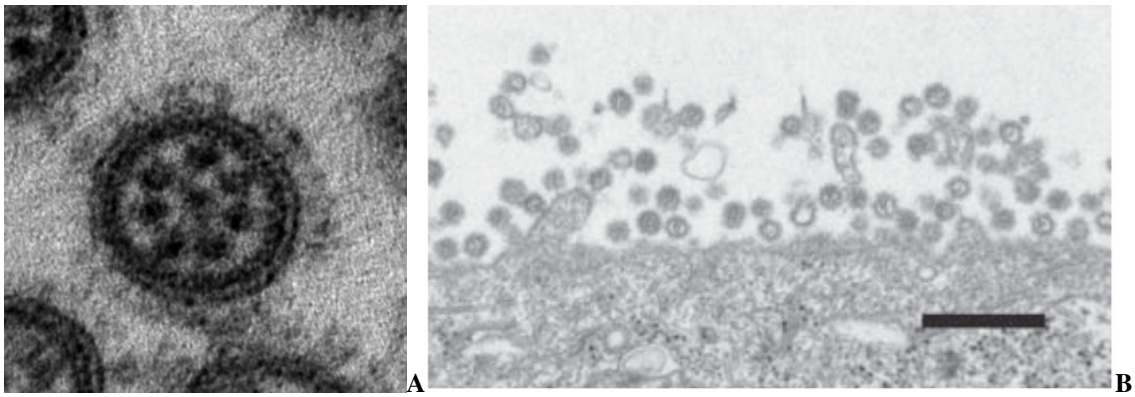


Figure 1 Viral particle (A) with the HA and NA spikes visible on the surface (diameter 100 nm) and budding (B) of influenza virus particles.
In (B) the electron micrograph thin-section image of the budding process from the apical surface of an infected cell is illustrated. Both figures adapted from (Wright 2007).

The second envelope glycoprotein NA has enzymatic activity, cleaving sialic acid residues from glycoproteins or glycolipids (Colman 1998). Sialic acid functions as a receptor for attachment of influenza virions and the neuraminidase activity of NA (cleaving such receptors) mediates the release of newly formed virus particles from the surface of infected cells (Hirst 1942; Palese 1976). The viral envelope contains a small number of copies of a third integral membrane protein, M2, which forms a tetramer with ion channel activity (Lamb 1985; Zebedee 1988; Hay 1998). M2 is involved in the infection process by modulating the pH within the virions, weakening the interaction between the viral ribonucleoproteins (RNPs) and the M1 protein.

The viral core contains the genome, which is organized in eight RNA segments (Figure 2). RNP complexes are composed of the RNA segments coated with nucleoprotein (NP), and associated with the polymerase complex (PB1 polymerase basic 1; PB2 polymerase basic 2; and PA polymerase acid) (Wright 2007). A layer of the matrix protein, M1 that is the

most abundant viral protein, surrounds the RNPs. The nuclear export protein (NEP/NS2) is associated with the viral RNPs (vRNPs) and the M1 protein (Richardson 1991). RNA segments 1 and 3 to 6 encode a single protein each. Segment 2 contains in addition to the PB1 protein, an alternate open reading frame (ORF) that gives rise to the polypeptide PB1-F2 (Chen 2001). Segment 7 encodes two proteins, the matrix 1 (M1) and matrix 2 (M2), with overlapping reading frames. Similarly, segment 8 encodes the nonstructural proteins NS1 and NEP/NS2, again with superimposed reading frames. NS1 is not present in virions, but it is abundant in infected cells.

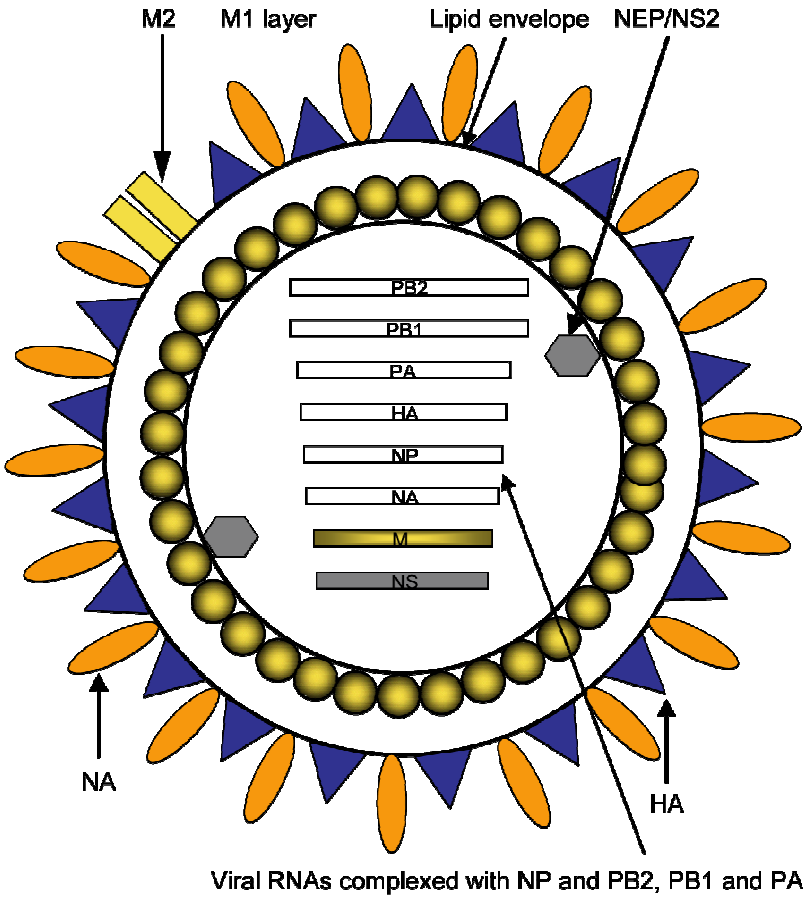


Figure 2 Schematic diagram of an influenza virus particle.

The HA (blue), NA (orange) and M2 (bright yellow) spike the viral envelope. Underlying M1 (dark yellow) proteins are associated with NEP/NS2 proteins (grey) and surround the vRNPs (rectangular). The eight genes are illustrated as rectangular boxes inside the virion with the longest gene on top (segment 1) and the shortest on the bottom (segment 8).

2.3. Viral replication

In humans, epithelial cells in the upper and lower respiratory tract are the primary target for influenza viruses. Host cell infection is initiated with binding of viral HA to sialic acid residues (neuraminic acids) located on glycoproteins or glycolipids on the cell surface (Colman 1998) (Figure 3). Receptor binding initiates the endocytosis by acidification inducing fusion of the viral envelope with the endosomal membrane and the release of vRNPs into the cytoplasm (Matlin 1981; Rust 2004; Smith 2004). The principle step of the fusion reaction is triggered by proton pumps within the endosomal membrane, which lower the pH inside the endosomes (pH 5-6). At low pH, a major conformational change in the HA protein is induced, resulting in the movement of the fusion peptide sequences of HA2, allowing their insertion into the target membrane (Carr 1993; Bullough 1994).

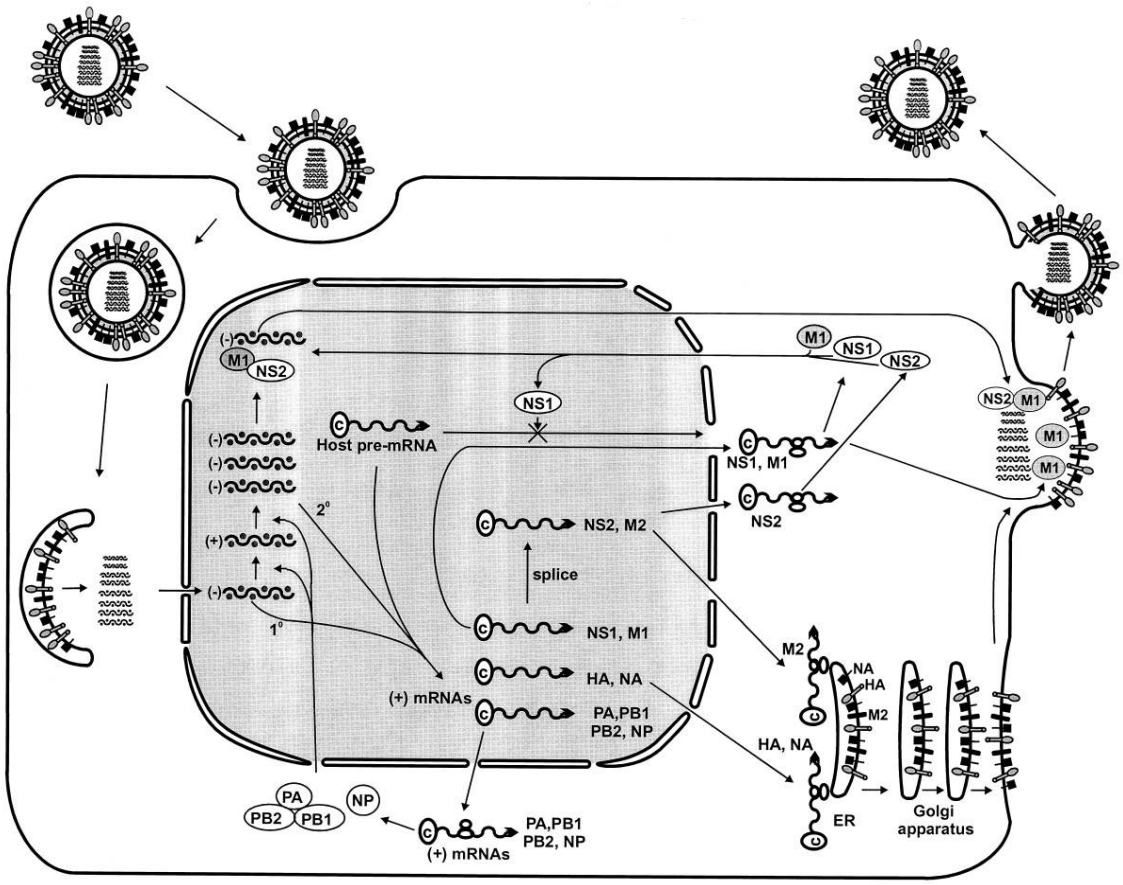


Figure 3 The life cycle of the influenza virus.

The virus enters the host cell by endocytosis (top left), followed by fusion and release of vRNPs (left). In the nucleus, translation and replication of the viral RNA takes place. Then, cytosolic ribosomal complexes (cytosol) transcribe mRNAs into viral proteins. The membrane proteins (HA, NA, M2) are transported

through the endoplasmic reticulum (ER), post-translationally modified and transported through the Golgi apparatus to the plasma membrane of the cell. The internal viral proteins (PB2, PB1, PA and NP) are packed into the viral particles in the cytosol. The budding process (top right) terminates when NA cleaves the sialic acid, thus releasing the virions from the host cell's surface. Adapted from (Palese 1976; Wright 2007).

After release of vRNPs into the cytosol, they migrate to the nucleus, where they act as a template for viral transcription. Negative-sense viral RNA (vRNA) is transcribed into mRNA by the transcriptase (consisting of PB1, PB2 and PA) carried with the RNPs (Wright 2007). The vRNA is replicated through a positive-sense intermediate, the complementary RNA (cRNA), which in turn is used as a replication template producing additional vRNA. The synthesis of proteins from viral mRNA is mediated by classical host protein translation in the cytosol. Viral RNPs are transported from the nucleus to the assembly site at the apical membrane of polarized cells (i.e. lung epithelial cells) (Rodriguez-Boulan 1978).

While viral envelope proteins HA, NA and M2 synthesis starts in the cytosol, polypeptide chains are glycosylated and folded into trimer and tetramers in the endoplasmatic reticulum (Braakman 1991; Doms 1993). Subsequently, the proteins are transported through the Golgi apparatus to the apical membrane. Synthesis and folding of the viral core proteins occur entirely in the cytosol. The packaging of RNPs into new virions is not a random process, but rather favors the formation of infectious particles (Fujii 2003; Noda 2006). Budding of completed viral particles is an active process and is mediated by the enzymatic activity of NA (Figure 1 B), which removes sialic acids from the surface of the host cell (Swayne 2003; Wright 2007).

2.4. Host species

Influenza A viruses infect a wide range of mammalian and avian species, including humans, birds, swine, horses, ferrets, whales and seals. Aquatic birds carry the majority of possible combinations of all known HA and NA subtypes and, thus are considered as the natural reservoir of influenza viruses (Table 1) (Easterday 1975; Alexander 2007). Stable lineages of the virus are established in domestic poultry, humans, pigs or horses (Webster 1992). In domestic poultry, certain subtypes become highly pathogenic and lead to substantial morbidity and mortality. Infections in humans can be lethal, but so far, no

HPAI virus with the ability of sustained human-to-human transmission has been reported (Wright 2007). Interestingly, the unique molecular configuration of sialic acid receptors on pig respiratory epithelia enables binding of influenza viruses derived from both human and avian species fostering its role as a 'mixing vessel' for influenza viruses (cf. Section 2.5 and 3.2) (Scholtissek 1985).

Table 1 Host species of influenza A virus subtypes, self-contained outbreaks of avian influenza in humans or pigs without sustained transmission are shown in parenthesis

HA subtype	Bird	Human	Swine	Horse
H1	H1Nx	H1N1	H1N1, H1N2	
H2	H2Nx	H2N2		
H3	H3Nx	H3N2	H3N2	H3N8
H4	H4Nx			
H5	H5Nx	(H5N1)	(H5N1)	
H6	H6Nx			
H7	H7Nx	(H7N7, H7N3)		H7N7
H8-H16	H8-H16Nx	(H9N2)		

2.5. Pathogenicity linked to the HA protein

Critical to viral pathogenicity, the HA protein is involved in viral attachment to a host cell and in subsequent fusion of viral and cellular membranes. To mediate entry of influenza viruses HA0 must be cleaved by a trypsin-like serine endoprotease at a specific site, normally coded by a single basic amino acid (usually arginine) between the HA1 and HA2 domains of the protein (Wilson 1981; Skehel 2000). After cleavage, the two disulfide-bonded protein domains produce the mature form of the protein subunits as a prerequisite for the conformational change necessary for fusion and hence viral infectivity (Section 2.3) (Carr 1993; Bullough 1994). The enzymes responsible for this mechanism are trypsin-like proteases, with a tissue distribution restricted to the respiratory tract, resulting in a local infection.

In most of the avian influenza viruses, the HA0 protein possesses a single basic cleavage site similar to that of human viruses, restricting the spread of these viruses. This mechanism alters when HA0 proteins of non-virulent or low pathogenic avian influenza (LPAI) change their amino acid sequence into a multibasic amino acid sequence as

observed for subtypes H5 and H7 of HPAI (Table 2). This permits cleavage of the HA0 protein by ubiquitously expressed proteases and often results in fatal systemic infection in poultry (Klenk 1994; Steinhauer 1999). During the 2003 outbreak of HPAI (H7N7) viruses in the Netherlands, 30 million birds succumbed to the disease or were culled to contain the spread (Elbers 2004). Several people in contact with the infected animals developed mild symptoms attributed to the disease. Lethal complications were observed in a single case of a veterinarian (Fouchier 2004; Koopmans 2004). While the latter virus has successfully been contained, the HPAI (H5N1) virus, which emerged in Asia 1997, spread worldwide, infecting more than 480 people with mortality rates up to 60% (WHO 2010b).

Table 2: Sequence at HA cleavage site of avian influenza viruses and their pathogenicity (Kawaoka 1984; Horimoto 1995; Garcia 1996; Xu 1999; Banks 2001; Suarez 2004; Chen 2005; Wright 2007; Owoade 2008)

Virus isolate	Subtype	Pathogenicity	Sequence at the HA cleavage site (*)
A/chicken/Pennsylvania/1/1983	H5N2	LPAI	PQKKKR*G
A/chicken/Queretaro/14588-19/95	H5N2	HPAI	PQRKRKTR*G
A/turkey/Italy/99 (consensus)	H7N1	LPAI	PEIPKGR*G
A/turkey/Italy/99 (consensus)	H7N1	HPAI	PEIPKGSRVRR*G
A/chicken/Chile/176822/02	H7N3	LPAI	PEKPKTR*G
A/chicken/Chile/4957/02	H7N3	HPAI	PEKPKTCSPLSRCRKTR*G
A/goose/Guangdong/1996	H5N1	HPAI	PQRERRRKKR*G
A/Viet Nam/DN-33/2004	H5N1	HPAI	PQRERRRKKR*G
A/chicken/Nigeria/OG2/2007	H5N1	HPAI	PQGERRRKKR*G

2.6. Host range restriction

The host species restriction of influenza viruses is characterized by multiple determinants including the receptor-binding specificity of the HA protein. Whereas most avian and equine viruses have a high binding affinity for sialic acid α2,3 linked galactose (SAα2,3Gal), human and classical H1N1 swine influenza viruses bind preferentially to SAα2,6Gal (Rogers 1983). In humans, SAα2,6αGal oligosaccharides are more frequent on non-ciliated epithelial cells of the upper respiratory tract, which are preferentially targeted and infected by human viruses. In contrast, the other receptor type, SA2,3αGal

oligosaccharides, are present on ciliated cells of the lower respiratory tract resulting in infections of broncheoli and alveoli (Matrosovich 2004; Shinya 2006).

Receptor distribution in the human respiratory tract may explain the increased infection rate by human strains, while avian virus infections probably require a higher dose and are therefore relatively rare. In avian hosts, such as ducks, SAα2,3Gal are found on epithelial cells of the intestine, preferentially infected by avian influenza viruses (Shortridge 2000). Specificity of host receptors is determined by aa that form the receptor-binding pocket. In HA proteins of subtpyes H2 and H3, glutamine at position 226 (Q226) and glycine at position 228 (G228) (both found in avian isolates) assigns preferential binding to SAα2,3Gal oligosaccharides present on avian epithelial cells. Leucine and serine at these positions (Q226L and G228S) in H2 and H3 viruses, promote binding to SAα2,6Gal receptor types of mammalian cells, enhance viral replication in the upper respiratory tract and facilitate transmission to humans (Connor 1994). For H1 viruses, aspartate (in human and swine viruses) or glutamate (in avian viruses) at position 190 determine preferential binding to SAα2,6 or SAα2,3 linkages, respectively (Matrosovich 2000; Kobasa 2004; Stevens 2004). In addition, the number and location of glycosylation sites are crucial for virus-host interactions (Claas 1998).

2.7. Pathogenicity triggered by NA, PB2 and NS proteins

The NA protein may also be involved in host range restriction and pathogenicity through promotion of viral spread within the respiratory tract by cleavage of sialic acids in the mucus (Palese 1974; Goto 1998; Peiris 2007). The NA activity of some avian viruses is more resistant to the low pH of the upper digestive tract than that of human- or swine-derived NA contributing to the host range restriction (Takahashi 2001).

Normally, avian viruses have glutamic acid in position 627 (E627) of PB2, however, a lysine 627 (E627K) correlates with replication in mammalian cells, reduced host defense and higher mortality in mice (Hatta 2001; Crescenzo-Chaigne 2002; Shinya 2004). This mutation is usually present in all human influenza strains, though, since 2001, an increasing number of HPAI (H5N1) viruses found in humans showed the residue K627 (Subbarao 1993a; Subbarao 1993b; Shinya 2004). Apart from the latter mutation, other aa

changes in PB2, PB1 and PA also interact with mammalian adaptation and virulence of HPAI viruses (Gabriel 2005; Li 2005; Salomon 2006).

The non-structural protein (NS1) is a multifunctional protein acting as an antagonist of the host cell antiviral response (Garcia-Sastre 2001; Katze 2002). NS1 protein is also involved in viral pathogenicity by limiting host cell responses at multiple stages (Yuen 1998; Wang 2000; To 2001). Notably, it targets both interferon (IFN-α/β) production and antiviral effects of IFN-induced proteins (Talon 2000; Ludwig 2002). Moreover, NS1 inhibits polyadenylation of cellular mRNA, thereby preventing its nuclear export. Specific translation is enhanced by NS1 in the cytoplasm, which leads to high loads of viral proteins, whereas cellular protein translation is decreased (whose cytoplasmic concentration is kept low by NS1) (Hale 2008). This mechanism contributes to the limitation of the host antiviral response by NS1.

3. The disease and the immune response

3.1. Virus transmission and clinical manifestations

Influenza spreads mainly through droplet or aerosol formation and can remain suspended in ambient air for a long time (Alford 1966). Transmission may also occur by contact with virus-contaminated hands or fomites; or with contaminated liquids, as the virus persists in water for 14 days at 4°C (Brown 2007). The incubation time for influenza ranges from one to five days, but the average is two days. In most cases, virus is found in specimens from nose and throat from one day before symptoms to four to five days after onset of disease. However, the level of virus shedding before symptoms is low and highest in the few days after symptoms start when the patient is feeling worse (Figure 4) (Richman 1976).

Influenza virus infection in the human respiratory tract directly targets the nasal and tracheobronchial epithelium, primarily of the upper and lower respiratory tract, and after virus replication provoking infected cells to undergo cellular apoptosis. The resulting loss of respiratory epithelial cells is one major reason for several of the clinical symptoms that characterize infection, such as cough, depressed tracheobronchial clearance and altered pulmonary function (Nicholson 2003). Although most symptoms overlap with other human respiratory infections, influenza disease usually presents as rapid onset of the

following combination of systemic and respiratory (both upper and lower) symptoms: high-grade fever, headache, muscle pain, runny nose, sore throat, non-productive cough and a degree of prostration (Potter 1998).

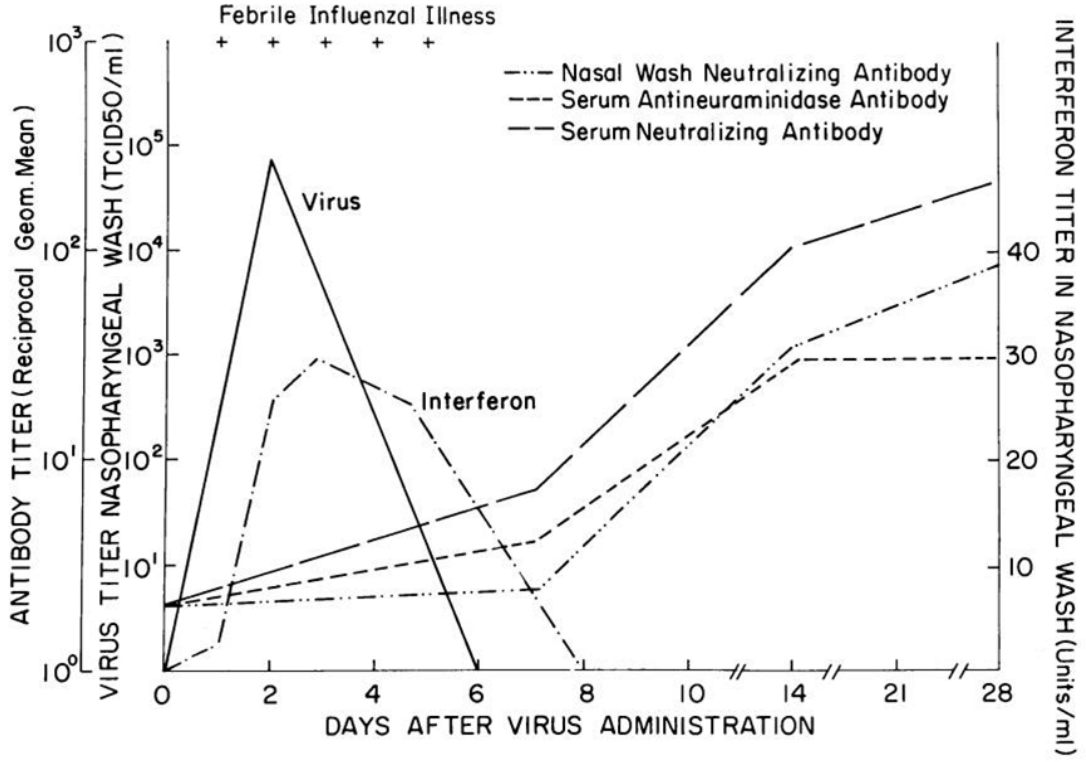


Figure 4 The pattern of virus replication.

Six seronegative volunteers had received $10^{4.0}$ $TCID_{50}$ of wild-type A/Bethesda/1015/68-like H3N2 virus intranasally on day 0, in relation to the onset of clinical symptoms, IFN response, and spout serum and nasal wash antibody responses. Adapted from (Richman 1976).

In severe cases, influenza infection leads to serious and potentially fatal complications. The age of the patient (the very young and the old), and the presence of chronic medical illness, such as cardiac and pulmonary, immunosuppression and pregnancy enhance the severity of outcome. Most commonly, influenza-associated pneumonia occurs with viral, bacterial or mixed viral-bacterial etiology (Cate 1987). Such patients can deteriorate rapidly with mortality reaching 50% (Ison 2002). Morbidity and mortality are highest in infants and children under two years of age. However, more than 90% of influenza-associated excess deaths occur in the elderly of 65 years and older (Janssens 2004).

3.2. Zoonotic influenza infections

Humans can naturally be infected with swine viruses leading to self-limiting diseases with mild symptoms that may suggest that mutational changes are required for transmission to and among humans (Kendal 1977; Wright 2007). In numerous reports the transmission of swine viruses to humans was described, some of these cases were fatal (Myers 2007). It was previously shown, that especially swine workers were at increased risk for influenza infections (Myers 2006). Swine can also be infected with human influenza viruses resulting in a serological reaction (Shope 1938; Castrucci 1994).

Humans have also been infected with HPAI (subtype H5N1) leading to severe influenza syndrome, with fever, cough, shortness of breath and pneumonia; additionally, gastrointestinal symptoms have been reported (Sandrock 2007; Hui 2008). In severe cases, the illness developed rapidly into bilateral pneumonia with acute respiratory distress symptoms requiring mechanical ventilation. Other complications included multi-organ failure and encephalitis (de Jong 2006a). Infections with HPAI of subtype H7N7 led to conjunctivitis in most cases and influenza-like illness (ILI) with one fatal case (Fouchier 2004). In China, some cases of infection with H9N2 avian viruses were reported without severe outcomes and without serological evidence of further human-to-human transmission (Peiris 1999).

3.3. Infection with HPAI (H5N1) in animals

Infections with LPAI viruses in animals normally present as mild, primarily respiratory disease (Webster 1992). However, in case of HPAI virus infections in poultry, sudden death without prior symptoms is common and morbidity and mortality often reach 90-100% within a few days. Birds that survive the first 48 hours develop respiratory distress, lacrimation, edema of the head and neck, sinusitis, and a comatose state (Alexander 2008). Mammalian species are also susceptible to HPAI infections. Carnivores (felids, dogs, mustelids, civets) naturally infected with subtype H5N1 suffered from respiratory distress, convulsions and death with multiple organ hemorrhages, necrosis and inflammation (Rimmelzwaan 2006).

3.4. Innate immune response

Infection elicits a cascade of host immune defenses leading to mucosal inflammation and stimulation of both the innate and adaptive immune response. Viral replication during the early stages of infection is controlled by the innate immune response (Durbin 2000). Influenza viruses are recognized by two Toll-like receptors (TLR3 and 7), which trigger intracellular cascades leading to innate resistance to infection (Diebold 2004). The release of cytokines attracts various immune cells, such as macrophages and natural killer cells into the respiratory mucosa (Julkunen 2000). The magnitude and pattern of cytokine responses in human influenza infection and the related responses to both time course of viral replication and clinical symptoms are illustrated in Figure 4. Within the first two days after infection, viral loads reach their peak and clear away from nasopharyngeal epithelia after about six days. The concentration of type I IFN-α/β, proinflamatory cytokines peak at day three post-infection and disappear at day eight post-infection (Hayden 1998).

While this response leads to the resolution of the infection and protection against reinfection, it is likely that it also contributes to the development of local and systemic symptoms as observed for infections with HPAI in humans (Hayden 1998). Immune pathology is thought to play an important role in H5N1 pathogenesis since high plasma levels of some cytokines (e.g. interleukin[IL]-6, IL-6; IL-8; IL-10; IFN-γ; tumor necrosis factor-α, TNF-α), macrophage- and neutrophil-attractant chemokines have been observed, predominantly in patients with fatal H5N1 subtype infection (To 2001; Peiris 2004; de Jong 2006b; La Gruta 2007; Abdel-Ghafar 2008; Hui 2009).

3.5. Adaptive immune response

The transition from innate to adaptive immune responses is triggered by stimulation of TLRs in endosomes of antigen-presenting cells (such as dendritic cells) (Diebold 2004). The antigen-presenting cells stimulate T lymphocytes such as T helper cells (mainly CD4-positives) and cytotoxic T lymphocytes (CTLs; mainly CD8-positives) that mediate both the cellular and the humoral immunity (Doherty 1997). CTLs contribute to the elimination of the infection by lysing virus-infected cells. B cell activation is mediated by cytokines released from T lymphocytes, and results in humoral immunity through production of

virus-specific antibodies (Lee 2005). In the effector phase of the adaptive immune response, secretory antibodies (immunoglobulin A, IgA) prevent infection at mucosal surfaces of the respiratory tract, while circulating antibodies (IgG) diffuse to and protect the lungs. The immunological memory for B cell response is lifelong, and subtype and strain specific. In contrast, T cell memory is more cross-reactive among different subtypes of influenza.

3.6. Neutralizing antibodies

Influenza strain-specific antibodies, produced during the adaptive immune response, represent the principal mechanism by which infection of cells is prevented (Murphy 1989; Skehel 2000). The main virus-neutralizing antibodies are directed against the viral surface protein HA, but antibodies against NA, NP and M proteins are also produced (Potter 1979). The level of serum antibody to HA and NA correlates with resistance to illness and with restriction of the influenza virus replication in the respiratory tract of humans (Clements 1986). HA antibodies neutralize the virus by blocking the receptor-binding site on the HA (Virelizier 1975), whereas NA antibodies mediate their antiviral effect primarily after the viral infection has been initiated by limiting virus release from the cell and hence restricting spread of virus within the respiratory tract of the host.

For influenza viruses, like for other viruses, there is a concept of original antigenic sin. This concept described by Francis and colleagues states that “the antibody-forming mechanisms appear to be oriented by the initial infections in childhood so that exposure later in life to antigenically related strains result in a progressive reinforcement of the primary antibody” (Davenport 1953; Francis 1955; Francis 1960). Recently this was confirmed by reproducible observations after immunization experiments in the mouse model (Kim 2009). Although immunity mediated by influenza virus infection can be long lived, reinfection with antigenically related influenza A viruses occurs, indicating that immunity induced by a single infection is incomplete (Sonoguchi 1986). On the one hand, there is a gradual diminution in the total amount of serum antibodies. On the other hand, after infection with a new influenza subtype, antibodies are generated that react with only a limited number of antigenic sites on the HA glycoprotein; whereas after several infections, antibodies are generated that have a broad range of specificities (Wang 1986).

3.7. Diagnosis

There are different diagnostic tests available for influenza including virus isolation, antigen or viral RNA detection, and serology, which vary in their specificity and sensitivity. Viral isolation is performed in cell culture, such as Madin-Darby Canine Kidney cells (MDCK), or in embryonated chicken eggs (WHO 2005). Hemagglutination is based on the ability of influenza virus to agglutinate erythrocytes through viral HA protein binding to sialic acid residues on the red blood cell surface. This property common for all influenza subtypes can be used as a rapid hemagglutination assay to determine viral presence after isolation in cells or eggs. In the hemagglutination inhibition (HAI) assay, reference antisera or monoclonal antibodies directed against one of the 16 different HAs and 9 NAs subtypes, are used to identify the influenza subtype of isolates and determine antigenic properties of viral variants (WHO 2005). Rapid tests for antigen detection in clinical specimens, like direct immunofluorescence, provide results within hours with lower sensitivities than viral culture (Szretter 2006). Viral RNA detection by reverse transcription polymerase chain reaction (RT-PCR), is performed to type and subtype influenza infections with high sensitivity, and allows quantification of viral loads (cf. Chapter III, Section 47.1) (Ward 2004). Serological methods include enzyme-linked immunosorbent assay (ELISA) for the detection of anti-influenza A virus IgG and IgM antibodies, HAI and virus neutralization inhibition assay (VN). In the VN, serum antibodies directed against viral HA proteins neutralize live virus and inhibit subsequent infection in the appropriate host system (e.g. MDCK cells) (Szretter 2006).

3.8. Treatment of influenza infections and antiviral resistance

Antiviral drugs active against influenza are adamantane derivates, like amantadine (trade name Symmetrel®) and rimamantadine (trade name Flumadine®) and the NA protein inhibitors zanamivir (trade name Relenza®) and oseltamivir (trade name Tamiflu®) (chemical structure illustrated in Figure 5). These drugs interfere in steps of the replication process, either at virus entry, virus assembly in the host cell or virus release from infected cells. Adamantane derivates inhibit the M2 proton ion channel, thus are inactive against influenza B and C, which lack the M2 channel (Wang 1993; Hayden 1999). Their use against influenza A has also been limited because of adverse side effects and the introduction of drug-resistant viruses (CDC 2008; NICE 2008). Drug-resistance is

conferred by nucleotide mutations leading to aa substitutions in the M2 protein, with the most common mutation of serine-to-asparagine change at position 31 (S31N) (Scholtissek 1979; Zaraket 2010). During the season 2003 to 2004, the first circulating influenza A viruses subtype H3N2 were amantadine-resistant, which then replaced current drug-sensitive virus variants (Zaraket 2010). Beginning 2006, amantadine-resistant influenza A viruses subtype H1N1 emerged in Asia, but these variants did not establish as main viral populations and were subsided by amantadine-susceptible variants of the same subtype in the winter season 2008 to 2009. In addition, all HPAI (H5N1) viruses are resistant to both M2 channel blockers (Li 2004).

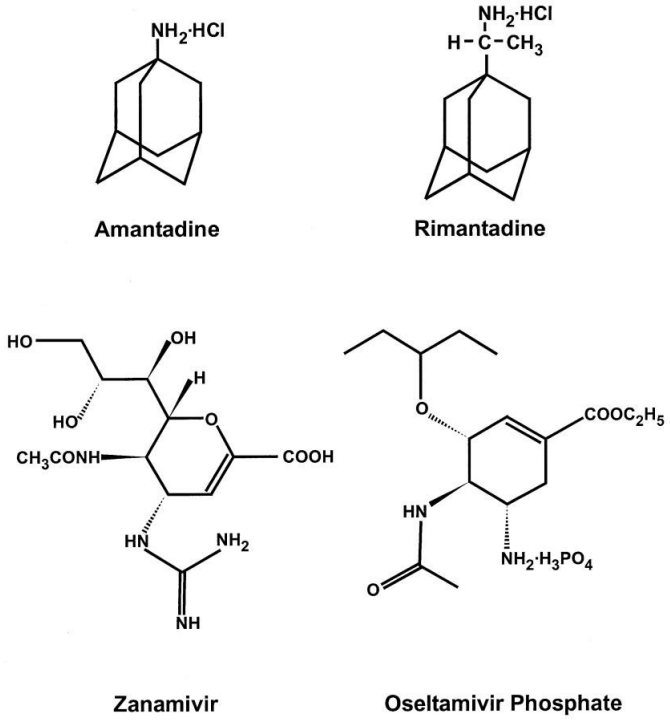


Figure 5 Structure of major anti-influenza compounds.
Top: M2 channel inhibitors, bottom: NA inhibitors. (Adapted from Wright 2007)

Neuraminidases cleave sialic acid residues on the cellular receptor that bind the newly formed virions to the cell and to one another (cf. Section 2.3). The neuraminidase inhibitors (NAIs) mimic NA's natural substrate and bind to the active site, preventing the enzyme from cleaving host-cell receptors (Colman 1994). NAIs are active against all nine NA subtypes of influenza A and against NA of influenza B viruses, having little toxicity. However, mutations in the effector sites of the NA protein have been identified conferring

resistance to NAIs. For oseltamivir, to fit in the active site, the aa must undergo a conformational change; mutations that prevent this rearrangement may lead to resistance. The oseltamivir resistance is conferred by a histidine-to-tyrosine mutation at position 275 (H275Y, N1 numbering) in the NA protein of N1 influenza subtypes (de Jong 2005). This mutation was firstly identified in viruses of a patient after treatment against infection with HPAI (H5N1) (Le 2005). During the season 2007 to 2008 oseltamivir-resistant virus variants of seasonal influenza subtype H1N1 emerged without selective drug-pressure, and the resistant strain seemed to be a natural, spontaneously arising variant (Moscona 2009). The latter drug-resistant variant subsequently spread worldwide, becoming predominant among circulating seasonal H1N1 viruses (CDC 2009a). In 2009, eventually a double-resistant influenza A H1N1 strain, resistant to both adamantine derivates and oseltamivir was detected in Hong Kong (Cheng 2009). The swine-origin pandemic A/H1N1 virus that emerged in 2009 is resistant against M2 channel inhibitors and remains susceptible towards NAI's. However, reports indicated sporadic cases of oseltamivir resistant variants without further transmission (WHO 2010a).

Table 3 Antiviral resistance testing

Results are shown on samples collected since September 1, 2009 from United States, WHO and National Respiratory and Enteric Virus Surveillance System collaborating laboratories and reported to CDC/Influenza Division were positive for influenza. Adapted from (CDC 2010)

	Samples tested (n)	**Resistant Viruses, Number (%)**	**Samples tested (n)**	**Resistant Viruses, Number (%)**	**Samples tested (n)**	**Resistant Viruses, Number (%)**
		Oseltamivir		**Zanamivir**		**Adamantanes**
Influenza A (H1N1)	1	1 (100.0)	0	0 (0)	1	0 (0)
Influenza A (H3N2)	13	0 (0)	0	0 (0)	18	18 (100.0)
Influenza B	23	0 (0)	0	0 (0)	N/A*	N/A*
2009 Influenza A (H1N1)	4,769	55†‡ (1.3)	1,855	0 (0)	1,858	1,854 (99.8)

*The adamantanes (amantadine and rimantadine) are not effective against influenza B viruses.
†Two screening tools were used to determine oseltamivir resistance: sequence analysis of viral genes or a neuraminidase inhibition assay.
‡Additional laboratories perform antiviral resistance testing and report their results to CDC. Three additional oseltamivir resistant 2009 influenza A (H1N1) virus has been identified by these laboratories since September 1, 2009, bringing the total number to 58.

3.9. Vaccines

Just a few years after the first isolation of influenza viruses in the 1930s, vaccine development began using the strain A/Puerto Rico/8/1934 (H1N1) that is still in use for reverse genetics influenza studies (Smith 1933; Fodor 1999; Neumann 1999). Today inactivated vaccines consist of split virus or subunit preparations, the latter containing only the isolated viral HA and NA proteins, which are produced from virus grown on embryonated chicken eggs (Wood 1998). Antigen present in the vaccine, mimic a natural immune response resulting in virus-neutralizing antibodies. Current seasonal influenza vaccines contain antigens from the two influenza A virus subtypes, H1N1 and H3N2, and a strain representing a current influenza B virus variant. To ensure an optimal antigenic match between the virus strains in the vaccine and the viruses circulating in the subsequent influenza season, the vaccine composition is adapted regularly and recommendations are made annually by the WHO (WHO 2010f). Numerous countries have implemented vaccination strategies to target risk groups including children, elderly, and immunocompromised. These vaccination efforts significantly reduced influenza-related respiratory illness, hospitalization rates and death among high risk patients for serious complications (Nichol 2003).

Pandemic vaccine development is based on a vaccine formula with adjuvant in order to minimize the dose of antigen required for induction of a protective immune response and thus to maximize an equitable distribution of vaccine doses (Lambert 2005). The vaccine against the pandemic H1N1 2009 was composed of inactivated split virus (only containing HA antigen) and an adjuvant, which differed depending on the manufacturer. For adjuvant MF59, efficacy was proven in an experimental vaccine containing subunits against H5N1 viruses before emergence of pandemic virus (Stephenson 2005).

4. The evolution and epidemiology

The epidemiology of influenza viruses is characterized by the viruses' constant antigenic variation to escape the host immune response. In contrast to most other respiratory viruses, influenza viruses possess two main mechanisms by which they change their antigenic properties, namely antigenic drift and antigenic shift. These allow them to reinfect humans among other host species and cause disease (Wright 2007). The antigenic evolution of

influenza viruses shapes the primary basis for recurring annual epidemics and occasional pandemics.

4.1. Antigenic drift and seasonality of human influenza

Due to the lack of proof-reading activity of RNA polymerases, point mutations occur more often in RNA than in DNA viruses (Domingo 1997). These high mutation rates lead to a variety of nonidentical, but closely related, mutant viral genomes in RNA viruses that are subjected to a continuous process of genetic variation, the so-called viral quasispecies (Domingo 2001; Domingo 2006). The continuous replication introduces mutations in the viral RNA genome of influenza that eventually modify aa, most often in the two major surface proteins, HA and NA. When these changes occur in epitopes of viral antigens they result in antigenic drift variants. The mutation frequency of influenza virus RNA estimates about one in 100'000 nucleotides; thus each new viral genome (14 kpb length) contains one or more mutations (Stech 1999). As a result, virus variants with such substitutions have a selective advantage over the original virus, since pre-existing antibodies against the viral HA neutralize newer drift variants of the virus less efficiently or not at all (Treanor 2004). These antigenic drift variants are the cause of new epidemics of seasonal influenza viruses and typically prevail for two to five years before being replaced by a different variant (Wright 2007).

In temperate climate zones, influenza epidemics occur almost exclusively in the winter months (October to April) on the Northern Hemisphere and (April to October) the Southern Hemisphere, while in tropical regions influenza is present all year round. However, the reasons for seasonal infection patterns are not yet completely resolved; the common assumption is that crowding in small spaces might be a factor (Wright 2007). Despite their annual seasonal character, influenza epidemics are unpredictable, since it is uncertain when they will start and how long they will last. Though much attention is paid to the impact of pandemics, many more people die in the intervening years because of the seasonal influenza epidemics than during the pandemics themselves (ECDC 2008).

4.2. Antigenic shift

Antigenic shift, the second important evolutionary mechanism, involves major antigenic changes through the introduction of new subtypes. The unique segmental organization of

the viral genome facilitates genetic reassortments that results from simultaneous infection of a host cell with two influenza A strains and is characterized by an exchange of gene segments during the reassembly of a new virus. Another major antigenic shift may occur after direct transmission of a new virus (mammalian or avian host) to humans or by reintroduction of an older strain into the population. The newly introduced proteins are immunologically distinct from the previously circulating strains and result in high infection rates in the naïve population, leading to global outbreaks of influenza pandemics. It has been hypothesized that influenza viruses of different species may also be involved in the co-infection, that would reassort and transmit readily from the human or other mammalian host, as has been assumed after the emergence of the pandemic H1N1 2009 virus (Figure 5) (Bouvier 2008).

4.3. Pandemics of the 20th century

Of the three major pandemics of the 20th century, the most dramatic was certainly the 'Spanish Flu' between 1918 and 1920 caused by influenza A virus of the subtype H1N1 with as many as 50 million estimated casualties (Johnson 2002). The eight viral gene segments were most closely related to avian-like H1N1 virus whose entire genome was transmitted and adapted subsequently to its new host (Gamblin 2004; Taubenberger 2005; Taubenberger 2006). In humans, new influenza strains with a mix of avian and human gene segments were successively introduced in 1957 (H2N2) and 1968 (H3N2), leading to the "Asian flu" and the "Hong Kong flu" pandemics. Each of the latter subtypes replaced the previous strains, and in 1977, the H1N1 subtype was reintroduced in the human population and is referred to as "Russian flu". This influenza H1N1 strain was identical in all eight gene segments to an H1N1 strain from 1957 (Nakajima 1978; Kilbourne 2006). Since then, both the H3N2 and H1N1 subtypes co-circulated (Neumann 2009).

In the first years of the new millennium, the annual epidemics have been mild compared to previous years. The usual experience after a pandemic is that the new pandemic strain dominates the annual epidemics for some years which are then more vigorous and severe than in the years before the pandemic (ECDC 2009). This hypothesis was confirmed by the early 2009 pandemic.

4.4. Pandemic of swine-origin influenza virus in 2009

The anticipated pandemic was thought to arrive via the avian host and being most likely of subtype H5N1. However, in April 2009, a previously unknown influenza A virus subtype H1N1 was isolated from humans in Mexico and in the US to eventually become the first pandemic strain of the 21st century. Shortly after initial cases were reported, the virus spread rapidly to geographically dispersed countries and across continents. As of May 25, 2010, the WHO reported laboratory confirmed cases of pandemic influenza H1N1 2009 in more than 214 countries with over 17919 deaths worldwide (WHO 2010e).

Phylogenic comparison of the complete viral genome sequence revealed swine origin. The virus contains a unique combination of gene segments derived from five different influenza viruses. The neuraminidase (subtype N1) and the matrix gene segment closely resembled Eurasian swine genetic lineages, (Figure 6, NA and M segment in pink), which were originally derived from an avian influenza virus and thought to have entered the European swine population in 1979 (Penseart 1981). Five gene segments were derived from a triple reassortant strain resulting from reassortment events between North American avian influenza (Figure 6, PB2 and PA segments in yellow), human H3N2 influenza (Figure 6, PB1 segment in blue) and classical swine influenza (Figure 6, NP, NS segments in green). Finally the hemagglutinin derived from the North American swine influenza subtype H1N2 (Figure 6, HA segment green). The pandemic virus origin is neither known nor could its first emergence be located, however, phylogenetic analysis revealed a probable circulation in humans beginning in September 2008 (Smith 2009b).

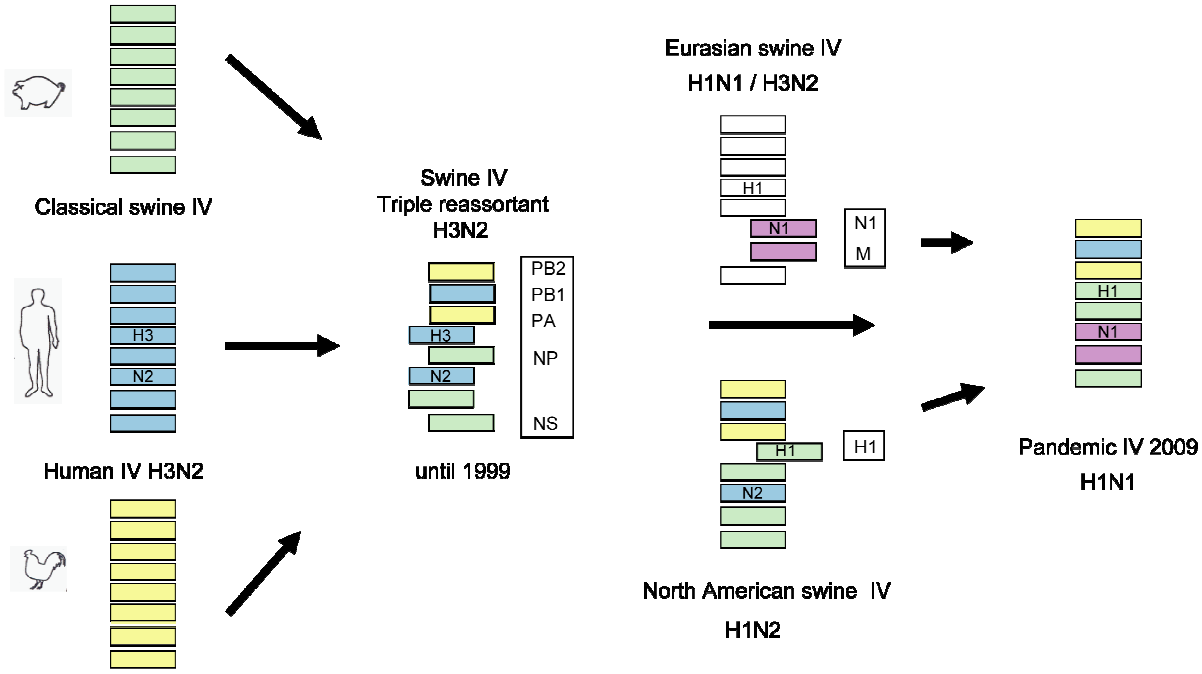


Figure 6 Molecular evolution of the human pandemic 2009 H1N1 influenza virus.
Viruses from three different host organisms and different IV subtypes were involved (left, avian genes in yellow, human in blue, classical swine in green). These gave rise to the pandemic virus (right) through an intermediate swine virus (triple reassortant, center) and with the contribution of N1 (NA gene subtype N1) and M genes from Eurasian swine IV and the H1 (HA gene subtype H1) from North American swine IV. Abbreviation: IV-influenza virus.

5. The evolution and geographic spread of HPAI (H5N1) viruses

5.1. Global epidemiology

The dynamics of viral evolutionary mechanisms are displayed in the epidemiology of HPAI (H5N1) outbreaks and subsequent spread. The first case was isolated from a child with fatal respiratory illness in Hong Kong in 1997 (Yuen 1998). Surveillance and epidemiological studies established that several avian influenza subtypes, including H5N1, co-circulated in chicken, ducks and geese on live bird markets in Hong Kong (Shortridge 1998). Intensive poultry culling in markets and farms contained the outbreaks and no new cases were found until 2000 (Shortridge 2000). The HPAI (H5N1) virus resulted probably from a reassortment between a H5N1-like virus (HA gene, A/goose/Guangdong/1/96), a H9N2-like virus (internal genes) and/or a H6N1-like virus (NA gene and/or internal genes) (Guan 1999; Hoffmann 2000).

Poultry culling did not interrupt the continuous circulation of Gs/Gd/96-like viruses, some of which reassorted with unknown viruses from an aquatic bird reservoir (Guan 2002b; Webster 2002). Several genotypes with distinct internal genes emerged in 2001 and 2002 causing a number of outbreaks in China (Guan 2002a; Guan 2004). One of these genotypes became dominant in Southern China and eventually differentiated into the distinct H5N1 clades that continue to circulate until today. During 2003, HPAI (H5N1) started to spread to other southeastern Asian countries (Republic of Korea, Thailand, Vietnam, Japan, Cambodia, Lao PDR, Indonesia, and Malaysia) with outbreaks in poultry and wild birds (FAO 2004; WHO 2009a).

In 2005, a large outbreak affected thousands of waterfowl at Qinghai Lake, an important breeding site for migratory birds in Western China. From the four genotypes detected one became dominant (Chen 2006; Peiris 2007). By the end of 2005, viruses with the same genotype were reported from Central Asia (Russia, Kazakhstan, Mongolia), Eastern Europe (Turkey, Romania, Croatia, Ukraine) and eventually, these viruses spread to more countries in Europe, the Middle East and Africa (WHO 2009a). After 2005, the outbreaks continued in Eastern, Southeastern, and Southern Asia, and phylogenetic analyses revealed the co-circulation of several genotypes, or clades. Although various gene constellations resulting from reassortments were observed, the HA gene was still derived from A/goose/Guangdong/1/96 H5N1-like virus evolving by genetic drift. This high genetic diversity led to the implementation of an international standard nomenclature for HPAI (H5N1) viruses (2008b).

Starting with the initial H5N1 strain A/goose/Guangdong/1/96 (clade 0), HPAI (H5N1) has now evolved into ten major clades (0 to 9) and additional subclades. The emergence of multiple clades and subclades in Asia reflects the uninterrupted circulation of H5N1 despite culling and vaccination measures (Chen 2008). In 2009, eleven Asian countries detected HPAI (H5N1) in wild birds, farms and live bird markets (OIE 2009). The continuous circulation can be explained by movement of birds between live bird markets and backyard farms, free ranging ducks as an interface between wild and domestic birds, and large waterfowl populations. Furthermore, legal or illegal bird movements and poor biosafety measures contribute to a favorable breeding ground for influenza viruses in Asia and beyond (Olsen 2006; Chen 2008).

5.2. African epidemiology

A seroprevalence study in sub-Saharan Africa conducted in commercial poultry in Nigeria between 1999 and 2004 did not detect antibodies to influenza viruses. Since Nigeria has the largest and the most active poultry industry in that region, this may suggest that at least LPAI viruses did not enzootically circulate in sub-Saharan poultry populations (FAO 2005; Owoade 2006). However, when HPAI (H5N1) spread from Asia across Russia to Europe, it also reached Africa. The first officially reported case occurred in commercial poultry farms in northern Nigeria in February 2006 and spread throughout most of the Nigerian Federal States (ProMED 2006). The genetic diversity, the timeline, the observed substitution rates, and the phylogenetic relationship suggested that three sublineages (A, B, and C) of the HPAI (H5N1) viruses of subclade 2.2 were independently introduced into the country (Ducatez 2006; Ducatez 2007a). Hemagglutinin gene sequences clustered with strains found in Europe, Russia, and Western China, but were distinct from strains identified in China and Southeast Asia (Ducatez 2006; Ducatez 2007b).

Within three months, outbreaks were reported in Egypt (sublineage B), Niger (sublineage A), Cameroon, Burkina Faso (sublineage C), Sudan (sublineage C), Côte d'Ivoire (sublineage C) and Djibouti (sublineage B) and all strains were most closely related to viruses found earlier in Nigeria (Ducatez 2006; De Benedictis 2007; Ducatez 2007a; Fusaro 2009). In 2007, H5N1 spread also to Ghana (sublineage C), Togo (sublineage A) and Benin (Cattoli 2009). The co-circulation of several sublineages led to multiple reassortment events between sublineage A and C viruses in Nigeria (Chapter IV, part 1, (Monne 2008). Reassortant strains ($AC_{HA/NS}$ reassortant, with HA and NS genes derived from sublineage C and the other six genes from sublineage A) from Benin were closely related to Nigerian reassortant from 2007 (Cattoli 2009). In 2008, only four African countries (Nigeria, Togo, Egypt and Benin) reported H5N1 outbreaks (OIE 2008; WHO 2009a) and the Nigerian HPAI (H5N1) virus, was phylogenetically most closely related to European strains (Fusaro 2009). In 2009, the only African country with endemic HPAI (H5N1) in domestic poultry was Egypt (WHO 2009a). The high diversity in viruses of sublineage B, primarily found in the country in 2006 (and in Nigeria in 2006), has been defined as a third-order clade 2.2.1 (2009).

6. Objectives

This work aims at determining the molecular characterization of influenza viruses and adaptive immune response mechanisms after infection with influenza viruses. Phylogenetic analyses and molecular epidemiology of full genome sequences target identification of putative reassortment events of avian and human viruses.

As described in the previous paragraphs, substantial work has been done in our laboratory to investigate viral epidemiology of HPAI (H5N1) in Nigeria. Although the virus has been contained in 2006, a year later, several new outbreaks of HPAI (H5N1) were detected in poultry flocks in the country. Thus, the first study focused on the molecular characterization and epidemiology of HPAI (H5N1) viruses collected in the southwest of Nigeria in 2007 (Chapter IV, part 1). Potential reassortment events were investigated using full genome sequencing and phylogenetic analyses to compare these new strains to earlier HPAI (H5N1) strains identified in other African countries and Eurasia. Moreover, special attention was paid to aa mutations in the proteins, which have the potential to alter pathogenicity or host range of HPAI viruses.

As previously mentioned, mutations in the binding regions of antiviral compounds can confer viral resistance. During the season 2007 to 2008, human influenza viruses of subtype H1N1 emerged, which were resistant to the NAI oseltamivir. Therefore, the second part of this work focused on surveillance of seasonal influenza of subtype H1N1 in Luxembourg during the winter season 2007 to 2008 to assess viral drug susceptibility. The investigations in this study aimed at linking clinical and epidemiological data such as prophylaxis, treatment, or stockpiling of oseltamivir to the emergence of genotypic resistant H1N1 viruses (Chapter IV, part 2). Additionally, the viral epidemiology and molecular characterization of drug-resistant and sensitive influenza strains was studied based on full-length genomic sequences. Using phylogenetic analysis potential genomic markers were identified that can differentiate oseltamivir-resistant from drug-sensitive viruses. Moreover, quasispecies or minor populations of drug-resistant viruses were investigated to hypothesize about their contribution to the emergence of the oseltamivir-resistant strains using pyrosequencing and cloning (Chapter IV, part 3).

After the emergence of a new human influenza virus in 2009, investigating the serological immunity against this virus became important. The virus was a result of reassortments of previous circulating swine viruses. Past studies showed that professional contact to pigs enhances the risk of infection with swine influenza viruses resulting in neutralizing antibodies that can cross-react with human influenza viruses. Thus, the fourth part explored humoral immunity against zoonotic infections with influenza viruses in humans. This work aimed at evaluating neutralizing antibody responses against the new pandemic influenza A/H1N1 virus and an avian-like swine influenza virus of the same subtype in individuals with professional contact to pigs in comparison to sera of the general population from Luxembourg (Chapter IV, part 4). In addition, epidemiological data were collected in a questionnaire in order to determine risk factors associated with zoonotic influenza infections.

Chapter II-Materials

1. Chemicals

Compound	Supplier
Agarose	Lonza
Ampicillin	Sigma
Bovine serum albumin (BSA) fraction V	Lonza
Dimethylsulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Invitrogen
dPBS w/o Calcium and Magnesium	Lonza
Eagle's minimal essential medium (EMEM) + Glutamine	Lonza
Ethanol 100%	Merck
Ethidium bromide	Invitrogen
Ethylendiaminetetraacetic acid (EDTA)	Biorad
Fetal bovine serum (FBS)	Lonza
First-Strand Buffer	Invitrogen
Fungizone	Lonza
Gentamycin	Sigma
Glycerol	Sigma
2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES)	Lonza
Kanamycin	Sigma
Luria broth base (LB)	Invitrogen
Magnesium chloride ($MgCl_2$)	Invitrogen
Magnesium sulfate ($MgSO_4$)	
Medium 199	Invitrogen
Nucleotides (dNTPs)	Invitrogen
Oligonucleotides/primers	Eurogentec
Ofloxacin hydrogenic chloric acid (Ofloxacin-HCL)	Sigma
Orange G	Invitrogen
PCR buffer without $MgCl_2$	Invitrogen
Penicillin G-Streptomycin	Sigma/Lonza
PicoGreen® 10 000X	Molecular Probes

Compound	Supplier
Polymyxin B	Sigma
Potassium chloride (KCl)	Merck
Sodium acetate	Merck
Sodium chloride (NaCl)	
Sodium hydroxide (NaOH)	Merck
Sucrose	Sigma
Sulfamethoxazole	Sigma
SYBR®Green™ nucleic acid stain	Molecular Probes
SYBR®Safe™ DNA Gel Stain	Invitrogen
Tris(hydroxymethyl)aminomethane (Tris)	Sigma
Trypan blue	Sigma

2. Buffers and solutions

Buffer	Reagent	Volume/concentration
DNA loading dye (6x)	Orange G	25 mg
	Sucrose (40%)	4 g
	ddH_2O, store at 4°C	up to 10 ml
Sodium acetate 3M	Sodium acetate	26.409 g
	ddH_2O	100 ml
	Adjust pH 5.2, autoclave	
TAE-buffer (50x)	Tris	2 M
	Sodium acetate	25 mM
	EDTA	0.5 M
	Adjust pH 7.8	
TE-buffer	Tris	2 M
	EDTA	1 mM
	Adjust pH 7.6	
PCR buffer w/o $MgCl_2$ 10x	Tris-HCl (pH 8.4)	200 mM
	KCl	500 mM
First-Strand Buffer 5x	Tris-HCl (pH 8.3)	250 mM
	KCl	375 mM
	$MgCl_2$	15 mM
Virus transport medium (VTM)	Medium 199	500 ml
	BSA/199	25 ml
	Penicillin G-Streptomycin 100x	10 ml
	Fungizone 100x	10 ml
	Penicillin G	540 mg

	Polymyxin B	123 mg
	Gentamyicin	125 mg
	Ofloxacin-HCL	30 mg
	Sulfamethoxazole	100 mg
	Filter sterilize, store at -20°C	
BSA/199	BSA	5 g
	Medium 199	10 ml
	Dissolve BSA, adjust pH, filter sterilize	

3. Enzymes

Platinum® Taq DNA polymerase	Invitrogen
RNaseOUT™ (Recombinant Ribonuclease Inhibitor)	Invitrogen
SuperScript™ III Reverse Transcriptase	Invitrogen
OneStep® RT-PCR Enzyme Mix (Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStarTaq DNA Polymerase)	Qiagen
L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-Trypsin	Sigma
Trypsin-EDTA	Lonza

4. DNA markers

1 kb plus DNA ladder™	Life Technologies

To determine the length of DNA fragments during agarose gel electrophoresis the 1 kb plus DNA ladder™, containing DNA fragments of the following sizes was used:

12000, 11000, 10000, 9000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1650, 1000, 850, 650, 500, 400, 300, 200, 100 bp.

5. Bacterial strain and media

E.coli One Shot® TOP10: This electrocompetent bacterial strain was provided with the TOPO TA Cloning® kit and was used for transfection with PCR product containing vectors by electroporation.

S.O.C. Medium (Invitrogen)	Tryptone	2%
	Yeast Extract	0.5%
	NaCl	10 mM
	KCl	2.5 mM
	$MgCl_2$	10 mM
	$MgSO_4$	10 mM
	Glucose	20 mM

Bacteria were grown in liquid LB medium containing 25 mg/l of Luria Broth base that was autoclaved at 121°C for 15 min. Growth plates for spreading bacteria were prepared with an autoclaved 32 mg/l LB agar medium. Transformed bacteria were selected by adding ampicillin (100 µg/ml) or kanamycin (30 µg/ml) to the liquid or solid medium.

6. Cells, growth media and viral media

MDCK epithelial cells: ATCC® Number: CCL-34™

Table 4 Composition of MDCK cell growth medium and viral growth medium

Medium	Reagent	Final Concentration
MDCK cell growth medium (CGM)	EMEM + Glutamine (500 ml)	1x
	FBS	10%
	Penicillin-Streptomycin	100 U/ml-100 µg/ml
	HEPES	25 mM
	BSA	0.2%
Viral growth medium (VGM)	EMEM + Glutamine (500 ml)	1x
	Penicillin-Streptomycin	100U/ml-100 µg/ml
	HEPES	25 mM
	BSA	0.2%
	TPCK-Trypsin	2 µg/ml
	Sterilize reagents by filtering through a 0.2 µm filter before adding to EMEM	

7. Kits

Viral RNA extraction

QIAamp® Viral RNA Mini kit	Qiagen
MagMAX™ AI/ND Viral RNA Isolation kit	Ambion

Polymerase chain reaction (PCR)

OneStep RT-PCR Kit	QIAGEN

Cloning

TOPO TA Cloning® kit	Invitrogen

Sequencing

Jet Quick PCR Purification Spin® kit	Genomed
Big Dye Terminator v3.1 Cycle Sequencing® kit	Applied Biosystems

Pyrosequencing

Binding, Washing and Annealing buffer	Biotage
Streptavidin Sepharose HP	GE Healthcare

8. Vector

The pCR®4-TOPO® vector, included as a linear molecule in the TOPO TA Cloning® kit, has 3' thymidine overhangs at the insertion site and several restriction and primer binding sites up- and downstream of the insertion site. The M13 sequence can be used to amplify the insert while the T3 and T7 sequences can be used for transcription by a T3 or T7 RNA polymerase. In addition, the vector encodes resistance genes for ampicillin and kanamycin as well as a P_{lac}/LacZ mechanism for insertion verification (Figure 7).

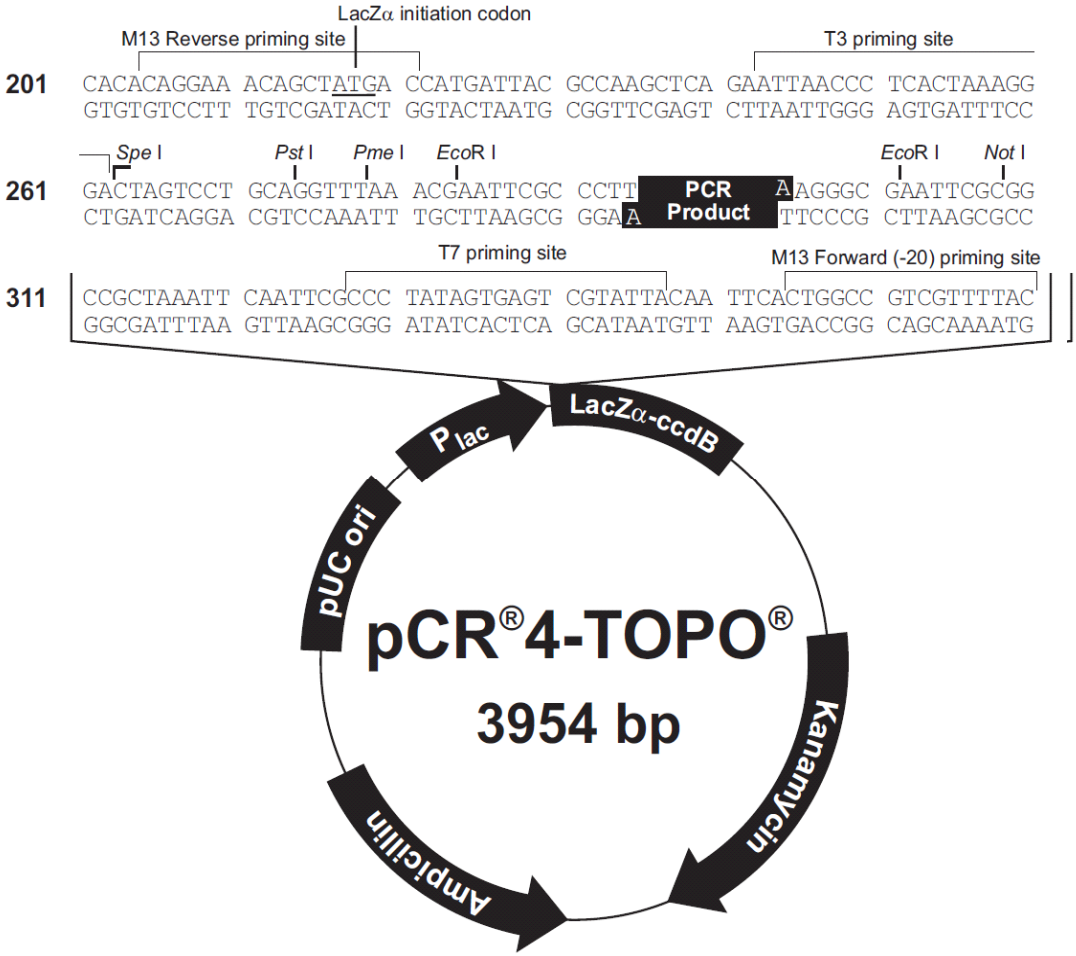


Figure 7. Map of the pCR®4-TOPO® cloning vector

Adapted figure from Invitrogen.

9. Primers

Detection of influenza A viruses

Table 5 Primer and probe sequences of TaqMan®-PCR conditions for the detection of influenza A viruses

Influenza virus (subtype)	Target gene	Primer (sense)	Primer 5'-3'sequence *	Nt- Position	Annealing °C
Influenza A virus (all subtypes)	M	Sense (For)	AAGACCAATCCTGTCACCTCTGA	134#	60°C
		Antisense (Rev)	CAAAGCGTCTACGCTGCAGTC	208#	
		Probe	TTTGTGTTCACGCTCACCGTGCC	174#	
Human influenza A (H1N1)	HA	1162 (For)	GAATAGCCCCACTACAATTGGGTAA	187#	58°C
		1163 (Rev)	GYAATTCGCATTCTGGGTTTCCT	233#	
		1164	AAGATCCAYCCGGCAACGCTGCA	214#	
Pandemic influenza A (H1N1)	HA	H1 SW (For)	CATTTGAAAGGTTTGAGATATTCCC	908§	58°C
		H1 SW (Rev)	ATGCTGCCGTTACACCTTTGT	970§	
		H1 SW	ACAAGTTCATGGCCCAATCATGACTCG	934§	

*For each TaqMan®-PCR reaction the two lines describe primers and the third line the probe with 5'-Fluorophore (6-FAM) and 3'-Quencher (TAMRA)
#According to the seasonal influenza A virus strain A/New Caledonia/20/1999 (H1N1)
§According to the pandemic A/H1N1 2009 influenza A virus A/Luxembourg/43/2009

Pyrosequencing for oseltamivir resistance mutation in NA

Table 6 Pyrosequencing primers for first round, nested PCR and pyrosequencing reaction

PCR	Primer (sense)	Primer 5'-3'sequence *	Position#	Annealing °C
1st round	N1-600Fh (For)	NGGAATTTCTGGTCCAGATGATGGA	600	58/56/54
	NA-1420 (Rev)	GGTGGGAGTAGAAACAAGGAGTTTTTT	1420	
nested	H1N1_pyro (For)	TAGAGTTGAATGCACCCAATTTT	800	60
	H1N1 pyro_bio (Rev)	*TCCCTGCATACACACATCACT	861	
Pyrosequencing reaction	H1N1_pyro_seq (For)	GAATGCACCCAATTTT	807	
	Sequence analyzed	YATTATGAGG	823	

#According to the seasonal H1N1 influenza A virus strain A/New Caledonia/20/1999
*5'-end labeled with dR Biotine

Random primers for reverse transcription of RNA into cDNA

The solution contains Random Primer oligonucleotides (hexamers), at a concentration of 0.09 optical density (OD) OD260 units/µl, in 3 mM Tris-HCl (pH 7.0), 0.2 mM EDTA (3 µg/µl; Invitrogen).

M13 primers for cloning verification PCR

Fragment	Primer	5'-3' sequence
M13	M13 Forward	GTAAAACGACGGCCAG
	M13 Reverse	CAGGAAACAGCTATGAC

10. Bioinformatics

The specific usage of the bioinformatical applications, in particular for phylogenetic analyses, is explained in Chapter III (cf. Section 10).

Primer design	FastPCR v3.7.8	(R. Kalender, University of Helsinki, Finland)
Sequence search with basic local alignment search tool (BLAST)	BLAST	(Altschul et al. 1990)
Sequence manipulations	BioEdit v.7.0.9.0	(Hall, 1999)
Distance calculations and neighbor-joining tree construction	MEGA 4.0	(Tamura et al. 2007).
Maximum likelihood tree calculation	PAUP* v4.0	(Swofford 2003)
Electropherogram analysis	SeqScape v2.5	Applied Biosystems
Sequence acquisition	Data Collection Software v3.0	Applied Biosystems
Sequence alignment	ClustalW v1.4	(Thompson, 1994)
Substitution model determination	Modeltest	Posada and Crandall 1998
	MrModeltest v2	Nylander, J. A. A. 2004 Evolutionary Biology Centre, Uppsala University
Real time PCR fluorescence acquisition and analysis	Opticon Monitor™ v3.1	Biorad
Pyrosequencing software	Biotage Pyromark	Biotage
Bayesian inferences of phylogeny	MrBayes software v3.1.2	Huelsenbeck and Ronquist 2001
Visualization of phylogenetic trees	TreeView v1.6.6	Page 2001
Other programs used		
TCID50 calculation	NCBI ID-50 v5.0	

SigmaStat v3.11 and SigmaPlot v9.01	Systat Software

11. Instruments

Centrifuges	Pico 17, Heraeus; Biofuge stratos®, Heraeus UNIVAP 150H, UniEquip
Balance	SARTORIUS Precision balance
Electrophoresis power supply	E835, Consort
Electroporation apparatus	Pulse Controller Plus, Capacity Extender Plus, Gene Pulser II Plus, Biorad
Fluorescence reader	GENios Plus, Tecan
Gel tank and casting form	Biozyme
Gel documentation system	InGenius, Syngene
Heating block	Thermomixer Comfort, Eppendorf
Incubator	HERAcell® 150, Heraeus
KingFisher Flex	Thermo Scientific, VWR
Microscope	Leica DMIL
PCR machine	Mastercycler® Gradient, Eppendorf
Pyrosequencer	Pyromark 96, Biotage
Real time PCR machines	Opticon® 2 DNA Engine, Chromo4™, CFX, MiniOpticon, Biorad ABI7500Fast, Applied Biosystems
Shaker	Multitron 2, INFORS-HT
Sequencer	ABI PRISM® 3130xl Genetic Analyzer, Applied Biosystems
UV transilluminator	Safe Imager™ 2.0 Blue-Light Transilluminator, Invitrogen
Vacuum Prep Workstation	Vacuum Prep Tool, Biotage
Vacuum source	Vacuum Pump, UNIequip
Vortex	Vortex-Genie® 2, Scientific Industries[1]

[1] Other company and product denominations mentioned in this document , such as: Invitrogen, Merck, Biorad, Sigma, Eurogentec, Moelcular Probes, Qiagen, Life Technologies, Abbott Diagnostics, Genomed, Applied Biosystems, Molecular Devices, Heraeus, UniEquip, Eppendorf, Tecan, Scientific Industries, Biozyme, Syngene, Biotage, Roche Diagnostics, may be trademarks or registered trademarks of their respective trademark owners.

Chapter III-Methods

The following methods were applied for data generation, collection and results applicable in Chapter IV. Exact concentrations of solutions, PCR protocols, and conditions mentioned in this chapter are described in detail in Chapter II (Materials). RNA extraction, influenza virus detection, subtyping, pyrosequencing and statistical analysis described in Chapter IV in part 2 were performed in the collaborating laboratories. Specific methods, such as description of specimens and clinical material or parameters of phylogenetic analyses, are specified in their respective parts in Chapter IV.

1. Culture of MDCK cells

MDCK cells are a host cell line for various viruses including influenza viruses. MDCK cells are adherent cells with elongated shape and divide on average one time in 24 hours. When cells cover 90% of the surface of a T75 cm^2 flask, the culture is splitted. MDCK cells were cultured under sterile biosafety level 1 conditions in a humidified 37°C atmosphere containing 5% carbon dioxide (CO_2). The medium (CGM) contained EMEM with Glutamine, FBS, BSA, HEPES and antibiotics (penicillin and streptomycin). The procedure for preparing an MDCK cell suspension with defined cell numbers is described for confluent T75 cm^2 flasks. If cell culture flasks of other sizes were used, the volumes were adjusted accordingly.

First, medium was decanted and 5 mL of pre-warmed to 37°C Trypsin-EDTA were added. The liquid was distributed over the entire cell sheet by gently rocking the flask for 1 minute and the Trypsin-EDTA was removed with a pipette and the same steps were repeated. Then 1 mL of the Trypsin-EDTA solution was added and the cell flask was incubated at 37°C until most cells detached from the surface (about 5-10 minutes). The flask was shaken or tapped whenever necessary to detach cells. Nine mL of CGM were added to inactivate the remaining Trypsin-EDTA before the cell suspension was transferred into a sterile 50 mL tube and centrifuged at 548 x g for 10 minutes at room

temperature in a Biofuge® Stratos (Heraeus). The supernatant was discarded and the cell pellet resuspended in 5 mL of CGM.

Before cell counting, 10 µL of cell suspension were mixed with 10 µL trypan blue solution (Sigma), a vital stain used to selectively color dead tissues or cells in blue. Then, cells were counted from 10 µL cell suspension in the improved Neubauer Hemacytometer and $2x10^6$ cells were seeded in a T75 cm^2 flask.

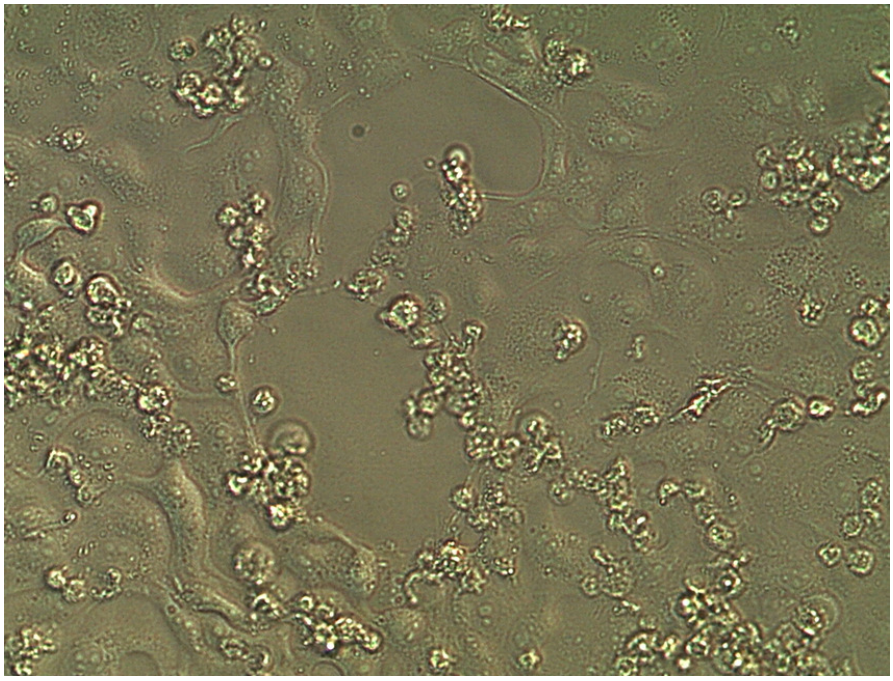


Figure 8 Example of cytopathic effect on MDCK monolayer infected with influenza A virus

2. Influenza A virus propagation and titration

Human, swine and avian influenza virus can be isolated and expanded on MDCK cells. The infectivity of the virus is dependent on the proteolytic cleavage of the HA0 protein into its two subunits HA1 and HA2, which enables the HA to undergo conformational change. This is essential for membrane fusion in the endosome and the release of the nucleic acid allowing for replication to proceed (cf. Chapter I, Section 2.5). Although MDCK cells lack such endogenous proteases, addition of exogenous trypsin to the medium is sufficient for proteolytic cleavage of the HA and the generation of infectious progeny. The trypsin used to propagate the virus is TPCK treated. FBS reduces infectivity and can also inhibit the activity of trypsin. The cytopathic effect (CPE) on MDCK cells can be observed in a bright field microscope by locating cell rounding and subsequent cell

death (Figure 8). Virus concentration should be determined by limiting dilution on MDCK cells. The highest dilution of virus still causing infection in 50% of the test is the tissue cell infectious dose ($TCID_{50}$) titer. All influenza virus experiments were carried out with personal protective equipment in rooms with negative pressure and biosafety level 3 conditions. In all experiments MDCK cells were incubated at 37°C in humidified atmosphere with 5% CO_2.

2.1. Influenza A virus propagation

One day before inoculation; $1.5x10^6$ cells were seeded in a T25 cm^2 flask to achieve a sub-confluent monolayer of MDCK cells 12-24 hours later. The cell monolayer was washed three times with 200 µL FBS-free virus growth medium (VGM) (containing TPCK-Trypsin). 200 µL of the specimen were inoculated on the cell monolayer and allowed to absorb for 30 minutes at 37°C. Then 6 mL of VGM were added and the CPE was observed daily. At 75% to 85% of CPE, the supernatant was centrifuged at 1370 x g for 5 minutes to remove excess cells and the supernatant was harvested in a stabilizing solution (BSA 0.5%). The supernatant was aliquoted and stored at –80°C. Presence of viral RNA was verified by influenza A virus TaqMan® real-time PCR from viral RNA extracted with one of the methods described in this Chapter in Section 4.

2.2. Influenza A virus titration

One day before the virus was titrated, $3x10^4$ cells per well were inoculated on a 96-well flat-bottom cell culture plate to ensure sub-confluent MDCK monolayer 12-24 hours later. First, the virus solution with unknown concentration was diluted ten times in VGM. A serial dilution of the latter virus solution was prepared on a sterile 96-well flat-bottom cell culture plate. Briefly, 120 µL of VGM were applied to all wells and 60 µL of the viral solution were added to all wells of column 1. Then, in column 1 the solution was mixed well five times by multichannel pipette uptake, then 60 µl were transferred to column 2, mixed and 60 µl were further transferred from column 2 to 3 and so on to column 11. With only VGM, column 12 was the negative control. The plate was covered and stored at 4°C until inoculation on monolayer. The cells were washed three times with FBS-free medium (VGM) and then 100 µL of the virus dilution were transferred from column 1 to column 12 of the MDCK cell plate. The plate was left for two hours at 37°C in the

incubator. After the incubation, the inoculums were removed with a multichannel pipette into a recipient containing virus decontamination solution and 200 µL VGM were added in each well. The plate was cultured for three days. The read-out was performed by CPE detection. Any well, which displayed CPE, was nominated as positive for viral growth and any well with an intact monolayer as negative for viral growth. The $TCID_{50}$ was calculated with the program ID-50 (Spouge).

3. Virus neutralization inhibition assay

In order to determine the neutralizing titer of serum, a limiting dilution of serum is mixed with a fixed concentration of influenza A virus in the VN. After a short incubation period, the mixture is added on a MDCK monolayer and incubated for three days. The influenza A virus is visually detected by CPE. Absence of damage to the monolayer indicates the presence of neutralizing antibodies in the serum. With limiting dilutions, the virus neutralizing titer can be determined.

To prepare the serum dilution all sera were heat-inactivated for 30 minutes at 56°C. Sixty µl of VGM were added to column 1-12 of a 96-well cell culture flat-bottom plate and 48 µl of VGM in wells A1 to A12. Then 12 µL heat-inactivated serum 1 were applied to wells A1-A4, serum 2 to A5-A8, serum 3 in A9-A12. A two step limiting dilution was performed starting with 60 µl from row A to row H and discarding the final 60 µl. To the serial serum dilution, 60 µL of virus solution with a defined $TCID_{50}$ of 120 were added to all wells. The virus-serum dilution plate was incubated for 2 hours. To ensure constant viral $TCID_{50}$ values, a 96-well plate was prepared in parallel as described in Section 2.2. Similarly, to $TCID_{50}$ determination, this assay was performed on sub-confluent MDCK cells on 96-well plates. After FBS-containing CGM was removed and cell monolayers were washed three times with 200 µL of VGM, 100 µL of virus-serum dilutions were transferred directly in each well of the MDCK cell monolayer. The plates were incubated for 3 days, followed by CPE read-out. Intact monolayers indicated the presence of neutralizing antibodies preventing viral growth; damaged or dead cells indicated absence of neutralizing antibodies in the serum. Neutralizing antibody titers were reported as the reciprocal of the highest dilution of serum completely neutralizing the viral growth. In all virus neutralization assays control sera were included which neutralized the viruses used.

4. RNA extraction

Viral RNA was extracted from human specimens (throat or nasal swabs of patients with acute respiratory tract infections [ARI] or influenza like illnesses [ILI]), from infected animal specimens (e.g. avian pharynx/tracheal and cloacal swabs) or supernatant from viral cultures using the QIAamp® Viral RNA Mini kit or the MagMAX™-96 AI/ND Viral RNA Isolation kit.

4.1. QIAamp® Viral RNA Mini kit

The manual extraction on spin columns was done following the manufacturers protocol. Briefly, 560 µL of Lysis Buffer (Buffer AVL) containing Carrier RNA (both included in kit) were added to 140 µL of the swab material, and mixed by vortexing and incubated at room temperature for 10 minutes. To this, 560 µL of 100% ethanol were added and mixed. The solution was briefly centrifuged to remove drops from the inside of the lid. The mixture was applied to a QIAamp® Mini spin column in a 2 mL centrifugation tube, which was then centrifuged for 1 minute at 6000 x g. The flowthrough was discarded and the column was washed once, first with AW1 Buffer followed by AW2 Buffer (included). RNA was then eluted from the column by adding 60 µL of distilled deionized water and centrifugation at 6000 x g for 1 minute. The eluted RNA was collected in a 1.5 mL microcentrifuge tube and stored at -80°C.

4.2. MagMAX™-96 AI/ND Viral RNA Isolation kit

In contrast to the QIAamp® Viral RNA Mini kit, the MagMAX™-96 AI/ND Viral RNA Isolation kit (Ambion) for rapid purification of Avian Influenza virus (AI) is not based on columns but on microspherical paramagnetic beads. These have a large available binding surface and can be fully dispersed in solution, allowing thorough nucleic acid binding (of viral RNA), washing, and elution. This protocol can be automated allowing a maximum of 96 samples in a single run, using the KingFisher Flex robot (Thermo scientific). This approach permits high RNA yields with low starting volumes of specimen.

The following protocol was used for RNA isolation of a maximum of 94 specimens in parallel. To ensure correct reaction conditions, volumes of buffers and solutions were calculated based on 100 samples. Five mL of Lysis Binding Solution concentrate were

mixed carefully with 100 µL of Carrier RNA and 5 mL of isopropanol were added and mixed well. To prepare the Beads Mix, 600 µL of Bead resuspension solution, 400 µL of nuclease free water and 400 µL of magnetic beads were mixed thoroughly. Then 600 µL isopropanol were added and mixed. To Wash Buffer 1, 35 mL isopropanol were added and to Wash Buffer 2, 80 mL of ethanol. To prepare the 96 well plates for the RNA extraction in the Kingfisher Flex Robot, the following reagents were pipetted: In plate 1, 100 µL Lysis Buffer and 20 µL of Bead Mix per well, plate 2, 100 µL per well of Wash Solution 1, plate 3, 100 µL per well of Wash Solution 2, plate 4, 100 µL per well of Wash Solution 2, plate 5, 50 µL per well of Elution Buffer. Fifty µL of the specimen were applied in plate 1 containing the Lysis buffer and the Bead Mix (well A1 to H12). A negative control was included every 12 wells (e.g. sterile virus transport medium [VTM] or PBS 1X). Specimens from the same animal (cloacal/tracheal swabs) were pooled and considered as one sample. The plates were placed in the KingFisher Flex Robot following manufacturer's protocol (position 1 to 5). Plate 5 was removed after finalization of the extraction procedure containing the eluted RNA, that was transferred into a 1.5 mL microcentrifuge tube and stored at -80°C.

5. Reverse transcription

The reverse transcription (RT) reaction, also called first strand cDNA synthesis, is a process in which reverse transcriptase creates single stranded complementary DNA (cDNA) from a single stranded RNA template. The reverse transcriptase enzyme (SuperScript® III) was used to synthesize first strand cDNA from 100 bp to >12 kb from viral RNA. Table 7 shows the reaction mixtures and steps of the RT with random primers. The resulting cDNA was diluted before it was used in subsequent PCRs.

Table 7. Reagents and conditions of reverse transcription reaction

Reagent	**End concentration**	
5 µL random primers	150 ng	
5 µL total RNA	(10 pg–5 µg)	
1 µL dNTP Mix	10 mM of each dATP, dGTP, dCTP and dTTP	
2 µL sterile, distilled H_2O		
Step 1	**Temperature (°C)**	**Time (min)**
Denaturation	72°C	10
Incubation	on ice (4°C)	1 (at least)

Reagent	End concentration	
4 µL First-Strand Buffer 5x	1x	
1 µL DTT	5 mM	
1 µL RNaseOUT™ Recombinant RNase Inhibitor	40 U/µL	
1 µL SuperScript® III	200 U/µL	
Step 2	**Temperature (°C)**	**Time (min)**
Incubation	25	5
Reverse transcription	50	80
Inactivation	70	10

6. Principle of the PCR

In the PCR DNA is amplified by a DNA polymerase resulting in double stranded PCR products, for example to quantify the presence of a specific DNA fragment, or to obtain highly concentrated DNA for downstream applications. The specificity of the amplification is determined by a set of oligonucleotides (referred to as primers), which only bind to a given sequence. The DNA dependent polymerase amplifies from the 5' end of the forward primer and towards the 3' end of the reverse primer during multiple PCR cycles. Each cycle consists of three steps conditional to specific temperatures.

Step 1) The denaturation step separates the double stranded DNA into single stranded molecules normally at temperatures between 92°C to 95°C.

Step 2) During the annealing step, the primers bind specifically to these single stranded molecules usually at temperatures between 50°C to 65°C.

Step 3) During the elongation step, the DNA polymerase copies the single stranded sequence between primers to a double stranded molecule, usually at a temperature of 72°C.

In certain applications, the annealing and the elongation steps are merged. The result of PCRs is verified by an agarose gel electrophoresis (cf. Section 7).

In a semi-nested PCR the PCR product of the first PCR is used as a template in a second PCR with one primer shifted (downstream for the forward primer or upstream for the reverse primer) and with the second primer unchanged. This approach increases the sensitivity of the amplification and, by shifting one primer, reduces the risk of amplifying unspecific products that could have appeared in the first PCR. The two PCRs are referred to as first round and semi-nested PCRs.

In contrast to that, nested PCRs are performed with two primers in the second PCR, which bind downstream of the forward and upstream of the reverse primer-binding site. With this approach, a higher specificity and sensitivity can be achieved. For applications such as virus detection, a specific primer design is essential due to high sequence variability among different strains of e.g. influenza A viruses.

In addition to the primers, template and the DNA polymerase PCR mixtures also contain dNTPs, the building blocks of DNA, magnesium chloride ($MgCl_2$), necessary for enzyme activity and primer binding, and the buffer, to ensure the correct ionic strength and pH for the PCR reaction.

The usage of a fluorescent molecule (SYBRGreen®, Invitrogen) binding to double stranded DNA, allows following the DNA amplification during each cycle of the PCR. These so-called real-time PCRs permit an easier quantification of template DNA and render consequent verification steps unnecessary. Another type of real-time PCR, referred to as 5' nuclease assay (or TaqMan® PCR) further increases specificity by using a third oligonucleotide (the probe), which is coupled to a fluorescent molecule and binds complementary to the sequence between the binding regions of forward and reverse primers. During the elongation step, the DNA polymerase separates the initially non-fluorescent molecule from the probe, which then becomes fluorescent. Thus, while SYBRGreen® fluoresces after binding to any double stranded DNA molecule, the fluorescence detected in a TaqMan® assay is linked to the amplification of a specific DNA sequence, thus effectively increasing the specificity of the PCR. TaqMan® assays are generally used for diagnostic and quantification purposes.

6.1. OneStep real-time TaqMan® for detection of influenza A viruses

The OneStep real-time PCR kit (Qiagen) consists of a RT step with specific primers followed by a specific detection PCR based on a TaqMan® assay performed in a single tube. In this assay, the primers and the fluorescently labeled probe targeted the matrix gene (M) of influenza A viruses (Chapter I, Section 2.2). Both, primers and probe sequences were based on a part of the M protein that is highly conserved among all influenza A virus subtypes forming all known combinations of H1-H16 and N1-N9 types

(Ward 2004). The PCR was performed on a real-time PCR cycler and the conditions are listed in Table 8.

Table 8 Conditions to prepare the influenza A virus detection PCR with the OneStep real-time PCR kit

Reagent	**End concentration**	**Volume in µL**
RT-PCR buffer 5x	1x	
MgCl2 (50 mM)	1.25 mM	
Sense (25 uM)	0.4 µM	
Antisense (25uM)	0.4 µM	
Probe (30uM)	0.3 µM	
dNTP mix (10 mM)	0.4 mM	
RNAse OUT (1 U/µL)	0.1 U	
OneStep RT-PCR enzype (1 U/µL)	1 U	
Template	RNA	2
Total		25
Step	**Temperature (°C)**	**Time (sec)**
Reverse transcription	50	1800
Denaturation & inactivation	95	900
Denaturation	95	10
Annealing & elongation	60	20
Repeat previous 2 steps 39 times		

6.2. Complete genome amplification of influenza A viruses

To amplify the complete genome of influenza A viruses, a premix of all PCR reagents excluding the specific primers was prepared following the conditions in Table 9. The specific primers were designed to amplify about 600-800 nucleotides of all eight influenza A virus gene segments resulting in overlapping PCR fragments. All PCRs were set up as first round and nested reactions with individual primer pairs. The PCRs were performed with the so-called touchdown protocol to enhance specificity of the PCR reaction and to ensure primer binding with decreasing annealing temperatures (Table 9).

Table 9 Conditions to prepare premix and parameters to perform touchdown PCR

Reagent	**End concentration**	**Volume (µL)**
PCR Buffer 10x	1x	
$MgCl_2$ (50 mM)	2.5 nM	
dNTP (10 mM)	200 nM	
SYBRGreen® 10'000x	80x	
Taq® DNA Polymerase	0.1 U/µL	
Primer For & Rev	0.5 µM	
Template	1:10 diluted cDNA	2.5
Volume		25
Step	**Temperature (°C)**	**Time (sec)**
Denaturation	95	30
Annealing	58, 56, 54	30
Elongation	72	60
Repeat the 3 previous steps 4 times at 58°C, 30 times at 56°C and 5 times at 54°C		
Final Elongation	72	600

6.3. Pyrosequencing PCR

Viral RNAs of a single influenza A virus specimen were screened for clones (minor populations) with single nucleotide polymorphisms (SNP) in the nucleotide position 823 in the neuraminidase (NA) gene. In a first PCR, a fragment of about 800 bp containing the SNP region of interest was amplified. In a second PCR (pyrosequencing PCR), a product of about 100 bp length was amplified with the forward primer binding downstream of the first primer binding region and the biotinylated reverse primer, binding upstream of the first primer. The PCR conditions were identical to the touchdown protocol (Table 9) for the first round PCR. For the pyrosequencing PCR, the parameters are shown in Table 10.

Table 10 Conditions and parameters for pyrosequencing PCR

Reagent	**End concentration**	
Premix with SYBRGreen®	(Table 9)	
Forward primer	750 nM	
Reverse primer	750 nM	
Template	cDNA or first round product 1:5 diluted	
Volume	50 µL	
Step	**Temperature (°C)**	**Time (s)**
Denaturation	95	30

Annealing	60	30
Elongation	72	60
Repeat the 3 previous steps 45 times		
Final Elongation	72	600

6.4. M13 PCR

To verify a successful cloning of the specific PCR product into plasmids, the inserted fragments were amplified by M13 PCR to be subsequently sequenced. The M13 primer binding sequences are located at the borders of the vector cloning site and allow for the amplification of the inserted fragment, independent of its sequence. A single bacterial colony serves as the template and is picked with a sterile wooden tooth pick from a growth plate and added directly into the PCR mixture. During the first denaturation step of the PCR, the bacterial cell wall is denaturized and plasmids are freed. The time of elongation depends on the length of the inserted fragment and generally is 1 minute for each 1000 bp shown in Table 11. M13 PCRs were run in a Mastercycler® Gradient (Eppendorf). Primers are listed in Chapter II Materials in Section 9.

Table 11 Conditions for the M13 PCR

Reagent	**End concentration**	
PCR Buffer	1X	
MgCl2	2.5 nM	
dNTP	200 nM	
M13 Forward primer	800 nM	
M13 Reverse primer	800 nM	
Taq® DNA polymerase	0.1 U/µL	
Template	Bacterial colony	
Volume	25 µL	
Step	**Temperature (°C)**	**Time (sec)**
Denaturation	95	20
Annealing	55	20
Elongation	72	variable
Repeat the 3 previous steps 45 times		
Final Elongation	72	600

7. Agarose gel electrophoresis

Visualization and separation of DNA products is done by agarose gel electrophoresis. DNA molecules are negatively charged due to their high content of phosphates, and migrate to the cathode when exposed to an electrical current (electrophoresis). To prepare a gel electrophoresis, the DNA of interest is pipetted into pockets or slots cut into an agarose gel immersed in an ion containing buffer (TAE buffer). When applying an electrical current to this buffer, ions including the DNA molecules, migrate through the tightly meshed structure of the agarose gel. Their progress through this mesh is size-dependent. It is visually followed by mixing the DNA with an ionic marker (Loading Dye 6x) which migrates similarly to a DNA molecule of 200 bp. The operator stops the migration when the marker has passed through approximately two-thirds of the gel. DNA products can be visualized under ultraviolet (UV) light by adding a molecule to the gel which fluoresces only when bound to double stranded DNA (SYBRSafe® DNA gel Stain, Invitrogen). By running a molecular weight marker or ladder (1 kb plus DNA ladder™, Invitrogen) in parallel with the DNA, the size of the PCR products can be evaluated by comparison to the known sizes of DNA fragments iof the marker.

Agarose gels (1.5%) were prepared by dissolving 1.5 g of powdered agarose in 100 mL of 1x TAE buffer using a microwave oven. Once completely dissolved, the mixture was allowed to cool to approximately 50-55°C, and 10 µL of SYBRGreen® were added and poured into the casting form (14 x 12 cm) and the comb(s) inserted (thickness of slots 1 mm). After 10 to 15 minutes, the agarose solidified, allowing the removal of the comb and the transfer of the gel to the gel chamber. The gel was covered with 1x TAE running buffer. Prior to loading DNA samples, 2 µL of 6x loading buffer were added to 5 µL of PCR product to prevent the PCR product from dissolving in the TAE buffer and to visually follow the migration. The gel was exposed to an electrical current of 130 V until the end of migration and images were taken after UV illumination at 300 nm wavelength with the InGenius Gel documentation system (Syngene). PCR products that presented single bands of the correct size were selected for further applications.

8. Cloning

Cloning can be used to identify the presence of multiple DNA populations in a single PCR product. The PCR product is mixed with vectors and an enzyme that ligates each DNA molecule into a single vector. While transfecting these vectors into bacteria, an antibiotic resistance is introduced to select successfully transfected bacteria. Since each bacterium can only contain one vector, it is possible to separate the vectors, and thus the DNA inserts, by spreading the bacterial mix on a growth plate. After an incubation period, colonies, which were initially based on a single bacterial cell, can be analyzed in downstream approaches.

The TOPO TA Cloning® kit (Invitrogen) contains linear pCR®4-TOPO® vector with 3' thymidine (T) overhangs and a topoisomerase covalently bound to the vector. While amplifying the Platinum® Taq DNA polymerase adds 5' adenosine (A) overhangs to every PCR product, which is used by the topoisomerase to introduce the PCR product into the vector and form a circular, closed plasmid. The vector pCR®4-TOPO® allows direct selection of recombinants via disruption of a lethal E. coli gene. Ligation of a PCR product disrupts expression of the gene permitting growth of only positive recombinants upon transformation in electrocompetent One-Shot® TOP10 bacteria (Invitrogen). Cells that contain a non-recombinant vector are killed upon plating. Specific sequences upstream and downstream of the inserted fragment are used in following applications such as sequencing, additional PCR reactions and restriction assays. Further vectors encode resistance genes to ampicillin and kanamycin as well as an origin of replication site (pUC-ori) necessary for maintenance in the bacterial cell (Chapter II, Figure 7).

For the cloning reaction, 4 µL of fresh PCR product were mixed to 1 µL of diluted salt solution and 1 µL of TOPO® vector (included in kit). Then, the reaction mix was incubated for 5 minutes at room temperature, and put on ice. TOP10 bacteria cells (Invitrogen) were 1:1 diluted in distilled water and 100 µL were mixed with 4 µL of the TOPO® cloning reaction in a 0.1 cm electroporation cuvette. The bacteria/vector mix was electroporated with following conditions: tension: 2.25 kV, resistance: 200 Ω, capacity: 25 µF, and immediately mixed to 250 µL of room temperature S.O.C. medium (Invitrogen). The bacterial suspension was incubated for 1 h at 37°C to allow expression of the

resistance genes. Then 60 µL of the suspension were spread on a LB solid agar growth plate containing kanamycin (or ampicillin). After 24 h incubation at 37°C bacterial colonies were picked with a sterile wooden toothpick and analyzed by M13 PCR.

9. Sequencing

9.1. Principle of Sanger sequencing

The sequencing method for de novo sequencing is based on the dye terminator method (Sanger sequencing). Extension is initiated at a specific site on the template DNA by short oligonucleotides (or primer) complementary to the template. The primer is extended with a DNA polymerase. Primer and DNA polymerase are mixed with dNTPs and fluorescently labeled, chain terminated di-deoxynucleotides at low concentrations. These lack a 3'-OH (Hydroxyl) group that is required for the formation of a phosphodiester bond between two nucleotides. Thus, these ddNTPs terminate DNA strand extension resulting in DNA fragments of varying length and varying terminal ddNTPs. Each fragment terminates with either ddATP, ddTTP, ddGTP or ddCTP labeled with fluorescent dyes, each with different wavelengths of fluorescence and emission. The fragments are size-separated by capillary electrophoresis in a polyacrylamide gel and at the end of the capillary; the fluorescence is read with a laser. Sequences are assembled by comparing the size dependent order of appearance of fragments and the nucleotide specific fluorescence peaks, referred to as sequence electropherograms. Before the sequencing PCR, all PCR products are separated from residual primers and non-incorporated nucleotides to avoid interference with the sequencing reaction. The purification is performed on DNA binding columns. Small fragments, such as primers, pass through the column while larger PCR fragments (80 bp-20 kbp) are bound. The quantity of DNA required for sequencing is between 1-3 ng for templates between 100 and 200 bp, 3-10 ng for 200-500 bp and 5-20 ng for 500-1000 bp.

9.2. DNA purification and quantification

The PCR purification was performed with the Jet Quick PCR purification Spin® kit (Genomed) as follows: 20 µL of PCR product were mixed with 140 µL of buffer H1 (included in kit) in a 1.5 mL microcentrifuge tube. The mixture was loaded on a Jet Quick Spin® column placed in a 2 mL centrifugation tube and centrifuged at 6000 x g. The

flowthrough was discarded and 500 µL of buffer H2 (included in kit) were added to the column, followed by a centrifugation as before. The spin column was transferred into a clean 1.5 mL microcentrifuge tube and 30 µL of TE buffer preheated to 70°C were added. After two minutes of incubation at room temperature and centrifugation as above, the eluted DNA was subsequently quantified.

The total amount of DNA in a solution was determined using Picogreen® (Invitrogen), a molecule able to bind to DNA and emitting fluorescence only in a bound state. First, 5 µL of the sample were diluted 20 times in 95 µL TE buffer and this volume was added to 100 µL of a Picogreen® solution (200 fold dilution of concentrated Picogreen® in TE buffer). A dilution series of DNA of known quantity mixed with Picogreen® served as a quantification standard. After mixing and incubating for 5 minutes at room temperature in the dark, the fluorescence was measured at a wavelength of 480 nm using the GENios® fluorescence reader (Tecan) with a 520 nm wavelength filter. The fluorescence values of the dilution series were plotted as a standard curve and a trend line (y=ax + b) was inferred. Based on the trend line equation, the quantity of DNA in the sample was determined.

9.3. **Sequencing reaction**

For the sequencing reaction 1 µL of BigDye Terminator® mix (Applied Biosystems) were added to 1.5 µL of 5x TE buffer, 1 µL of 5 µM primers and the necessary quantity of DNA, diluted in deionised water (max 5 µL). The mixture was adjusted to a volume of 10 µL with deionised water. Depending on the number of samples to be sequenced, the experiment was performed on 96 well sequencing plates. The PCR conditions were as follows: initial denaturation step 96°C for 3 minutes, 29 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for two minutes.

Non-incorporated dye was removed before sequencing for each sample by adding 5 µL of 125 mM EDTA and 10 mM of deionised water. After mixing, 60 µL of 100% ethanol was added to each well; the plate was vortexed and incubated for 15 minutes at room temperature in the dark. The plate was then centrifuged at 4°C, 1370 x g for 30 minutes in a Biofuge® Stratos (Heraeus). The ethanol was immediately removed by inverting the plate on tissue paper with a subsequent centrifugation at 456 x g for 60 seconds. The previous steps were repeated with 70% ethanol and centrifugation at 4°C for 15 minutes.

Then, the plate was dried for 15 minutes in a UNIVAP 150 H (UniEquip), and stored at 4°C until use. Preparation for sequencing consisted of heating the samples for 5 minutes to 95°C, adding 10 µL of HI-DI (Applied Biosystems) and incubation at 95°C for 5 minutes. The plate was loaded on the capillary sequencer (ABI PRISM®, 3130xl Genetic Analyzer, Applied Biosystems). All applications used capillaries with a length of 80 cm. Data was recorded as electropherograms.

9.4. **Principle of pyrosequencing**

In contrast to capillary sequencing, pyrosequencing is a DNA sequencing technique that is based on the detection of pyrophosphate (PPi) released during DNA synthesis. In a cascade of enzymatic reactions, visible light is generated proportionally to the number of incorporated nucleotides. The general principle behind different pyrosequencing reaction systems is that a polymerase catalyzes incorporation of nucleotide(s) into a nucleic acid chain. As a result of the incorporation, a pyrophosphate (PPi) molecule(s) is released and subsequently converted to ATP, by ATP sulfurylase. Light is produced in the luciferase reaction during which a luciferin molecule is oxidized.

$$(NA)n + Nucleotide \longrightarrow (NA)n+1 + PPi$$

$$PPi + APS \longrightarrow ATP + SO_4^{2-}$$

$$ATP + Luciferin + O_2 \longrightarrow AMP + PPi + Oxyluciferin + CO_2 + Light$$

Generated light is directed to the CCD (charge-coupled device) camera using a lens array located below the microtiter plate and data is recorded as pyrograms and is illustrated in Figure 9. These pyrograms can be analyzed and edited by Biotage Pyromark ID software. For analysis of SNPs by pyrosequencing, the 3'-end of a primer is designed to hybridize one or a few bases before the polymorphic position.

In order to perform DNA analysis using pyrosequencing technology, PCR products have to be processed to yield single-stranded DNA to which a sequencing primer can be annealed. The method to generate DNA template for pyrosequencing analysis produces high quality DNA from crude PCR reactions without prior purification. The PCR product, with a 5'-biotinylated strand, is captured on streptavidin-coated beads. Subsequently, the non-biotinylated DNA strand is removed by alkali treatment and separated from the

biotinylated strand attached to the bead. This washing step also serves to neutralize the pH. Finally, the beads with attached DNA are transferred to buffer containing the sequencing primer and the primer is annealed to the single-stranded template by heating and cooling. The strand released from the beads can also be used for pyrosequencing analysis, after proper neutralization.

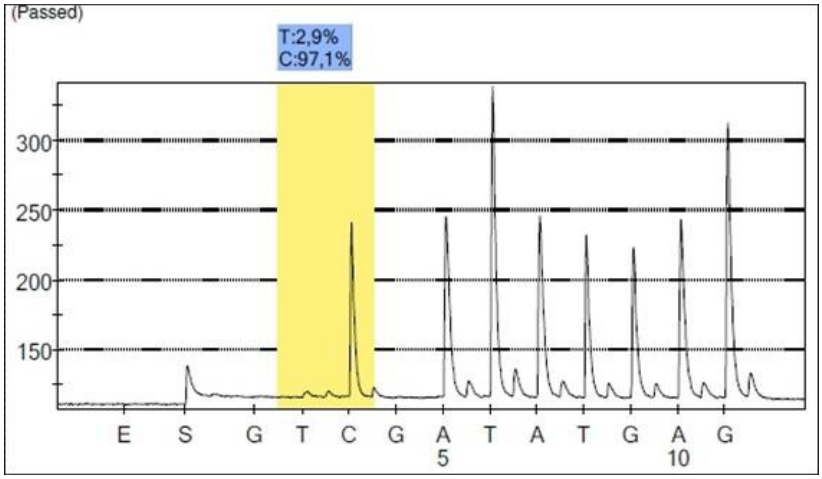


Figure 9. Pyrogram of a specific SNP in the NA gene in nucleotide position 823 of seasonal influenza A virus.

Above the specific SNP the proportions of nucleotide T or C are indicated in percent values. On the x-axis the nucleotide sequence is shown and on the y-axis the light intensity.

9.5. Pyrosequencing of SNP in the NA protein

To analyze the specific SNP at nucleotide position 823 of the NA gene of the seasonal human influenza A virus (H1N1), a first round PCR was performed to amplify a fragment containing the region of interest. In the following nested PCR, the forward primer together and the 5'-biotinylated primer were used for amplification of a 100 bp PCR product. After quantification of the PCR products (described in Section 9.2) they were processed as follows (calculated for 50 samples).

First, the pyrosequencing primer was diluted in 2500 µL annealing buffer to achieve final concentration of 4 µM. Then, 40 µL of the primer dilution were added to the wells of the pyrosequencing plate (96-well format). Meanwhile, the pyrosequencing plate support was preheated to 80°C. To prepare the binding buffer, 200 µL of streptavidin-coated beads were diluted in 2467 µL binding buffer and thoroughly mixed. All PCR products were adjusted to an optimal concentration of 200 ng in 40 µL, mixed with 40 µL of streptavidin-

coated beads solution, and incubated at room temperature on a shaker at maximum speed (or 14000 rounds per minute). In the meantime, substrate mix, enzyme mix, dATP, dGTP, dCTP and dTTP were added to the cartridge with volumes proportional to the number of samples and the sequence length. To purify the biotinylated DNA into the troughs of the vacuum Prep Workstation the following reagents were added. First, 180 mL ethanol (70%) in trough 1, 120 mL of denaturation solution (sodium hydroxide 1M) in trough 2, 180 mL washing buffer (in the pyrosequencing kit) in trough 3 and lastly, 180 mL high purity water were filled in trough 4. After applying the vacuum to the workstation, the probes on the vacuum tool were washed 20 seconds in high purity water by lowering the device in the washing trough. Then, the beads were captured by lowering the tool slowly into the PCR plate for maximal 3 minutes after the agitation was terminated. The vacuum tool was put into the trough 1, 2 and 3 and left in each for 5 seconds. The vacuum tool was turned 180 degrees in a horizontal position for 5 seconds (allowing complete aspiration of the washing buffer) and returned to the previous horizontal position. Before immersion into the pyrosequencing plate the vacuum was switched off to release the beads into the plate. The purified biotinylated DNA was then annealed with the primer by heating it at 95°C for 5 minutes and the plate and cartridge placed in the pyrosequencer. After finalization of the run, the Pyromark Software generated pyrograms.

10. Phylogenetic analysis

10.1. Electropherogram analysis and sequence alignments

Sequences were extracted from electropherograms by SeqScape® software (Applied Biosystems) and visually checked for inconsistencies. The software assembled individual sequences to complete genes by alignment to a known reference sequence. Sequences were imported into the sequence manipulation software BioEdit (Hall 1999) and further aligned to a set of reference sequences using the internal ClustalW algorithm (Thompson 1994).

A pair of sequences can be aligned by writing one sequence above the other in such a way as to maximize the number of residues (nucleotides or aa) that match by introducing gaps (spaces) into one or the other sequence (Figure 10). These sequence alignments consist of a set of sequences, which are known to be linked by function or genome location, where

differences are minimized. Sequences are similar when at least 50% of nucleotide positions are identical. For instance influenza A virus sequence similarities depend on the gene segment and subtype. Influenza viruses of subtypes H1 to H16 differ by 30% in their hemagglutinin gene nucleotide homology (Webster 2006).

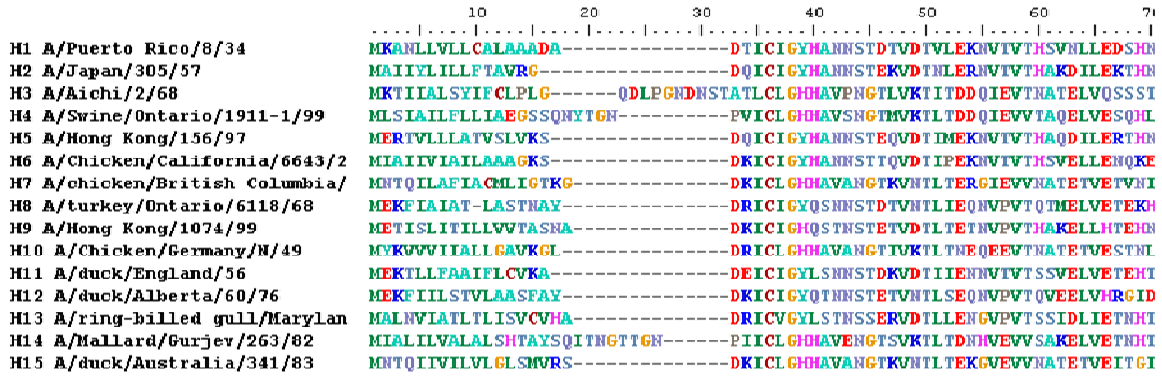


Figure 10. Amino acid sequence alignment of the HA protein of influenza A virus subtypes H1-H15 at positions 1 to 70.

Amino acids are represented in single-letter code with different colors and gaps as -.

10.2. Nucleotide distance calculations and models

Number of nucleotide (or aa) substitutions occurring between them usually measures the evolutionary distance between a pair of sequences. Evolutionary distances are fundamental for the study of molecular evolution and are useful for phylogenetic reconstructions and the estimation of divergence times. The nucleotide distance is the number of sites at which the two compared sequences differ. Evolutionary mechanisms are reversions that are series of mutations that restore the original nucleotide at a position. Kimura's two parameter model (1980) corrects for multiple hits, taking into account transitional and transversional substitution rates, while assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites (Kimura 1980). This is one of the most widely used models. Kimura two parameter distance calculations were conducted using MEGA version 4 (Tamura 2007).

10.3. Constructing phylogenies

A phylogenetic tree is a hierarchical structure consisting of nodes connected by branches. Nodes can either be external, as the tips of the tree of the taxa being considered or internal

nodes, as the points that represent a common ancestor of two or more other nodes. In a phylogenetic tree, sequences are linked by nodes which represent hypothetical ancestors or points of deviation between two sequences and connected by horizontal lines (branch is identical to the length proportional to the genetic distance, Figure 11). Sequences are derived from the same phylogenetic group when they cluster on the same node. Depending on the organism, phylogenetic groups are referred to as lineages, sublineages, genotypes, subtypes, families, genera or species.

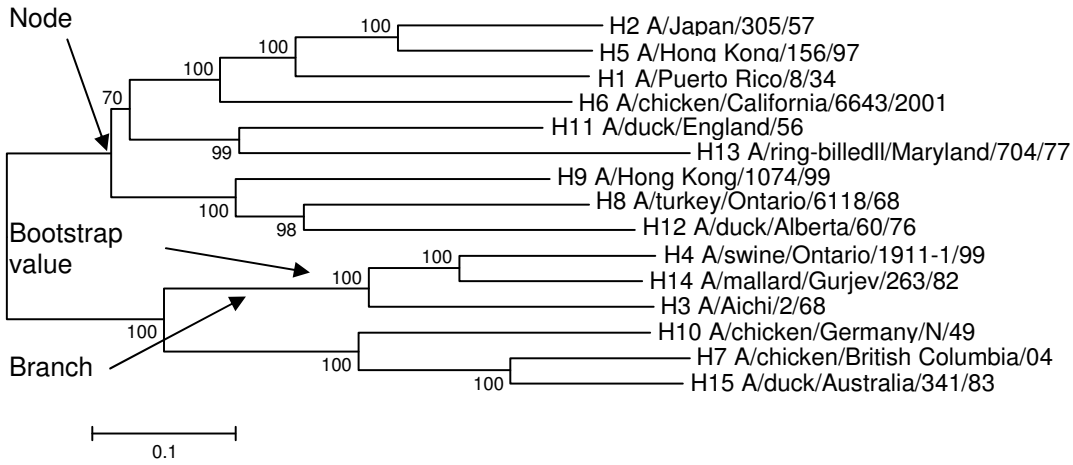


Figure 11 Phylogenetic tree of hemagglutinin genes of influenza A subtypes H1-H15.

Currently there are two methods for constructing phylogenies from protein and nucleic acid sequence alignments that are grouped according to the data they use, discrete character based states or distance matrix of pairwise dissimilarities. The character-state methods can use any set of discrete characters such as sequence data and are called Maximum likelihood (ML) or Bayesian inference (Hall 2005b). In contrast, distance-matrix methods start by calculating some measure of the dissimilarity of each pair of sequences to produce pairwise distance matrix, and then infer the phylogenetic relationship of the sequences from that matrix such as the neighbor-joining (NJ) method (Saitou 1987). This is the most commonly used algorithm for the tree phylogeny. The NJ method is a simplified version of the minimum evolution method, which uses distance measures to correct for multiple hits at the same sites, and chooses a topology showing the smallest value of the sum of all branches as an estimate of the correct tree.

However, more complex models of tree reconstruction are character-based; all use the multiple alignments directly by comparing characters within each column (each site) in the alignment. Maximum Likelihood tries to infer an evolutionary tree by finding that tree that maximizes the probability of observing the data. For sequences, the data is the alignment of nucleotides or amino acids. Bayesian analysis is a recent variation of Maximum Likelihood (Ronquist 2003). Instead of seeking the tree that maximizes the likelihood of observing the data, it seeks those trees with the greatest likelihoods given the data. Instead of producing a single tree, Bayesian analysis produces a set of trees of roughly equal likelihoods. The result of a Bayesian analysis are interpreted on the basis of the frequency of a given clade in a set of trees that is virtually identical to the probability of that clade (Hall 2005a).

10.4. Branch support

Several measures are used to assess the certainty of a tree or its branches. The support of branches is often assessed by employing statistical principles. The most widely used approach is bootstrapping (Felsenstein 1985). The bootstrapping method takes a subsample of the sites in an alignment and creates a tree based on those subsamples. That process is iterated multiple times (a typical number is 1000) and the results are compiled to allow an estimate of the reliability of a particular grouping. Those values are expressed as percentages, where high bootstrap values indicate significant nodes (e.g. >95%), and values under a certain cut-off are insignificant and the node can be ignored (e.g. <75%).
All trees sampled during a Bayesian analysis are usually summarized in a consensus tree. The Monte Carlo Markov Chain method samples trees from a distribution of probabilities which in turn is used to derive approximate posterior probabilities for each of split or clade (Ronquist 2003). The values are interpreted in a similar way to bootstrap values, with 1.0 representing 100% probability of a correct node in that position and with values lower than 0.75 (equal to 75%) being unlikely the correct position of the node.

10.5. Distance calculations and phylogeny of influenza virus sequences

Sequence analyses were performed with simple models of distance calculations followed by more complex approaches using ML and Bayesian inference. To ensure optimal performance fitting best to the data set and reliable estimates of evolutionary relation,

sequence alignments were compared with at least 32 different distance models using Modeltest and MrModeltest (Posada 1998; Nylander 2004). The model with highest likelihood was chosen for phylogenetic inference. Tree reliability was calculated by bootstrapping for ML analysis and by posterior probability for Bayesian analysis. The exact parameters of distance calculation models and tree phylogenies are explained in detail in the Chapter IV.

Chapter IV-Results and Discussions

Part 1

Results of part 1 were published as:

Gerloff NA, Owoade AA, Ducatez MD, Taiwo JO, Kremer JR, Muller CP. (2008) Replacement of sublineages of avian influenza (H5N1) by reasssortments, Sub-Saharan Africa. Emerging Infectious Diseases. 14:1731-35

Replacement of sublineages of avian influenza (H5N1) by reassortments in sub-Saharan Africa

Highly pathogenic avian influenza (HPAI) virus subtype H5N1 in Africa was first reported from northern Nigeria in February 2006. Phylogenetic analysis of the complete genome showed that these viruses were clearly distinct from the two lineages that were found during the same period in southwestern Nigeria (Ducatez 2006; Ducatez 2007a). The three sublineages (referred to as A, B, and C), two of which emerged from a common node, had evolved from subtype H5N1 strains that were originally found around Qinghai Lake in 2005. These strains clustered with viruses isolated from 2006 from southern Russia, Europe, and the Middle East (clade 2.2, www.who.int/csr/disease/influenza/tree_large.pdf) but not with the strains prevalent in southeast Asia (Salzberg 2007). The timeline, the observed influenza A (H5N1) substitution rates in Africa, and the phylogenetic relationship suggested that the sublineages were independently introduced into the country (Ducatez 2006; Ducatez 2007a). These sublineages were later found throughout Africa with a distinct geographic distribution (Ducatez 2007a; Ducatez 2007b). Sublineage A was also found in Niger and Togo (HA sequence); sublineage B was detected in Egypt and in a human patient in Djibouti (partial HA sequence), and sublineage C was found in Burkina Faso, Sudan, Côte d'Ivoire, Ghana (HA and NA sequences) (Macken 2001) and Cameroon (NA sequence) (Njouom 2008). Sublineage A strains were also referred to as European-Middle Eastern-African 2 (EMA 2), and both sublineages B and C belong to EMA 1 (Salzberg 2007). In 2006, one strain with reassorted genes was reported among 35 full-length sequences of the European–Middle Eastern–African lineage (Ducatez 2006; Ducatez 2007a; Ducatez 2007b; Salzberg 2007). This study describes new HPAI (H5N1) strains collected in southwestern Nigeria during the second half of 2007, most of which were different reassortants of sublineages A and C.

1. Specimen collection, virus extraction and phylogenetic analysis

Cloacal swabs were obtained from eight chicken farms in Lagos (n=1), Ogun (n=5), Oyo (n=1) and Ekiti (n=1) States in southwestern Nigeria from June through November 2007. RNA extraction from cloacal swabs, RT–PCR amplification, and gene sequencing were

conducted as described in Chapter III. For most viruses, complete sequences were obtained for all gene segments. Kimura two parameter distances were calculated based on complete or partial gene sequences by including the maximum sequence length available from all strains included in the comparison. Phylogenetic trees were calculated by using PAUP version 4.0 beta 10 (Wilgenbusch 2003) with the Maximum Likelihood method. The best model was determined by using MODELTEST (Posada 1998). Trees were visualized in TreeView (Page 2001).

2. Results

2.1. Reassortants

All genes of A/chicken/NIE/EKI15/2007 and A/chicken/NIE/OYO14/2007 clustered phylogenetically with sublineage A strains. The Kimura distances between the genes of these viruses were 0.4%–1.4%. Among all subtype H5N1 virus sequences published in the Influenza Sequence Database (Macken 2001), NIE/EKI15/2007 and NIE/OYO14/2007 gene sequences were most closely related to those found throughout 2006 and 2007 in Nigeria. Thus, these viruses have most probably evolved from a sublineage A virus initially imported into the country in 2006. This finding is also corroborated by published substitution rates from Africa (Ducatez 2007a).

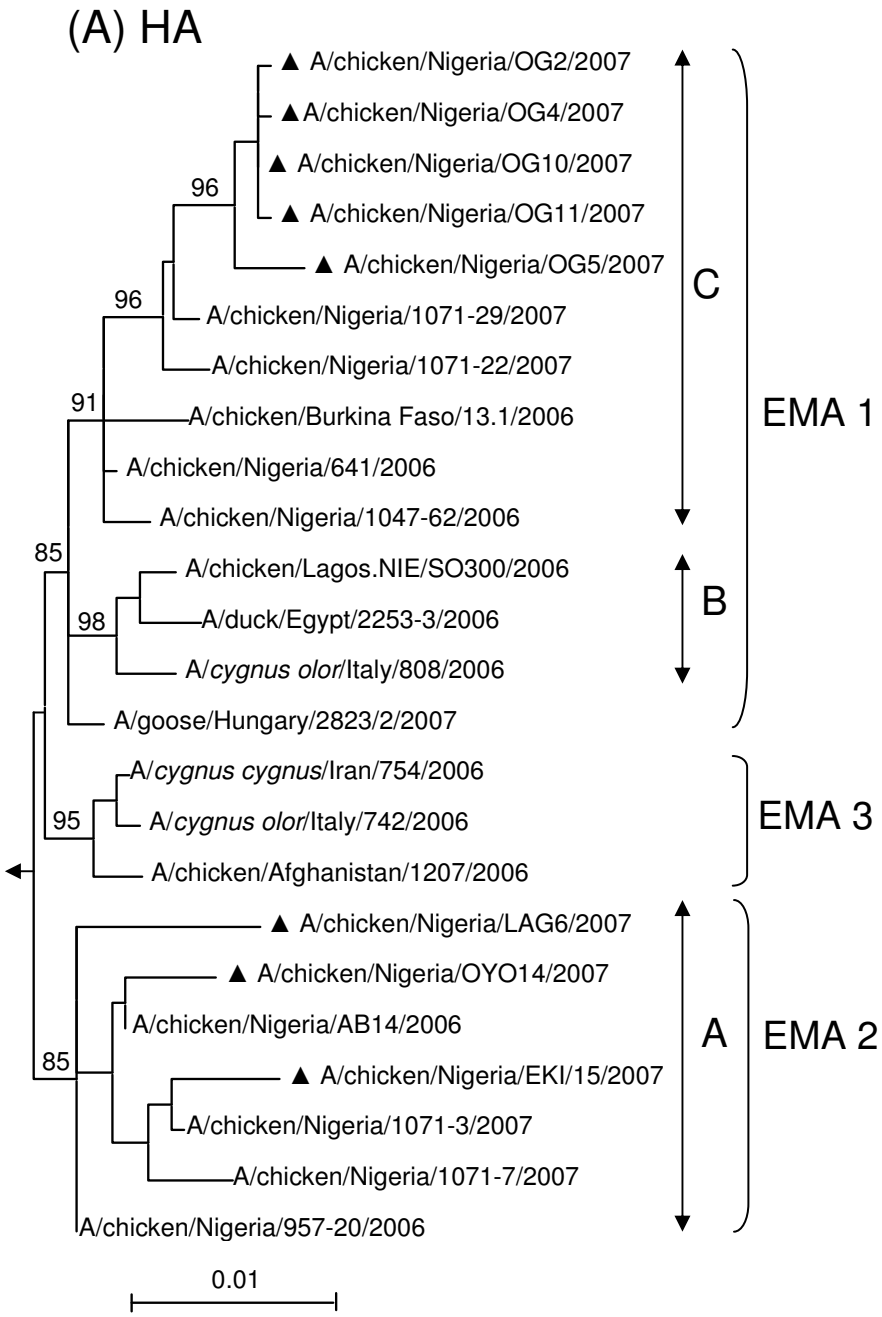
Five viruses had HA and NS genes grouping with sublineage C virus genes, whereas the other gene segments were most closely related to sublineage A viruses (e.g., A/chicken/NIE/OG2/2007 and OG5/2007, Figure 12, Figure 13). These viruses evolved by reassortment from sublineages A and C viruses ($AC_{HA/NS}$ reassortment, Figure 2). Another virus (A/chicken/NIE/LAG6/2007) also showed evidence of reassortment between sublineage A and sublineage C. However, in this virus only the NS gene belonged to sublineage C (Figure 12, Figure 13). The other seven gene segments of A/chicken/NIE/LAG6/2007 were derived from sublineage A (AC_{NS} reassortant).

2.2. Reassortments between Reassortants

Four of the $AC_{HA/NS}$ reassortants (A/chicken/NIE/OG2/2007, A/chicken/NIE/OG4/2007, A/chicken/NIE/OG10/2007, and A/chicken/NIE/OG11/2007), all of which were from Ogun State, had similar sequences in all genes (Kimura distances 0%–0.7 %). The AC_{NS} reassortant A/chicken/NIE/LAG6/2007, obtained from a chicken farm in Lagos State,

diverged by 0.9% in the complete NS gene (derived from C lineage), and by 0.7% to 1.4% in sublineage A–related gene segments from the latter four $AC_{HA/NS}$ reassortants. Some gene segments of the $AC_{HA/NS}$ reassortant A/chicken/NIE/OG5/2007 were most closely related to the other four $AC_{HA/NS}$ reassortants, whereas, other gene segments were closer to the AC_{NS} reassortant A/chicken/NIE/LAG6/2007. Matrix protein, HA, NS, NA, and NP genes of NIE/OG5/2007 showed a maximal Kimura distance of only <0.4% to $AC_{HA/NS}$ reassortant genes but a distance of 0.6%–1.5% to the AC_{NS} reassortant (A/chicken/NIE/LAG6/2007).

In contrast, PB2, PB1, and PA genes were more closely related to the AC_{NS} reassortant (maximum Kimura distance <0.6%) than to $AC_{HA/NS}$ reassortants (minimum Kimura distance for the different genes 0.7%–0.8%). For instance, A/chicken/NIE/OG5/2007 differed by 12 nucleotides in the PA gene from the most closely related $AC_{HA/NS}$ reassortant (A/chicken/NIE/OG2/2007) but by only one nucleotide from the AC_{NS} reassortant (A/chicken/NIE/LAG6/2007). On the other hand, A/chicken/NIE/OG5/2007 had 15 nucleotides in the NP gene different from the A/chicken/NIE/LAG6/2007 but only one nucleotide difference compared with the closest $AC_{HA/NS}$ reassortant (A/chicken/NIE/OG11/2007) (Figure 13). This finding strongly suggests that A/chicken/NIE/OG5/2007 is the result of an additional reassortment event involving an exchange of genes between the $AC_{HA/NS}$ and AC_{NS} reassorted viruses.


(A) HA
▲ A/chicken/Nigeria/OG2/2007
▲A/chicken/Nigeria/OG4/2007
▲ A/chicken/Nigeria/OG10/2007
▲ A/chicken/Nigeria/OG11/2007
▲ A/chicken/Nigeria/OG5/2007
A/chicken/Nigeria/1071-29/2007
A/chicken/Nigeria/1071-22/2007
A/chicken/Burkina Faso/13.1/2006
A/chicken/Nigeria/641/2006
A/chicken/Nigeria/1047-62/2006
A/chicken/Lagos.NIE/SO300/2006
A/duck/Egypt/2253-3/2006
A/*cygnus olor*/Italy/808/2006
A/goose/Hungary/2823/2/2007
A/*cygnus cygnus*/Iran/754/2006
A/*cygnus olor*/Italy/742/2006
A/chicken/Afghanistan/1207/2006
▲ A/chicken/Nigeria/LAG6/2007
▲ A/chicken/Nigeria/OYO14/2007
A/chicken/Nigeria/AB14/2006
▲ A/chicken/Nigeria/EKI/15/2007
A/chicken/Nigeria/1071-3/2007
A/chicken/Nigeria/1071-7/2007
A/chicken/Nigeria/957-20/2006
96
96
91
85
98
95
85
C
B
A
EMA 1
EMA 3
EMA 2
0.01

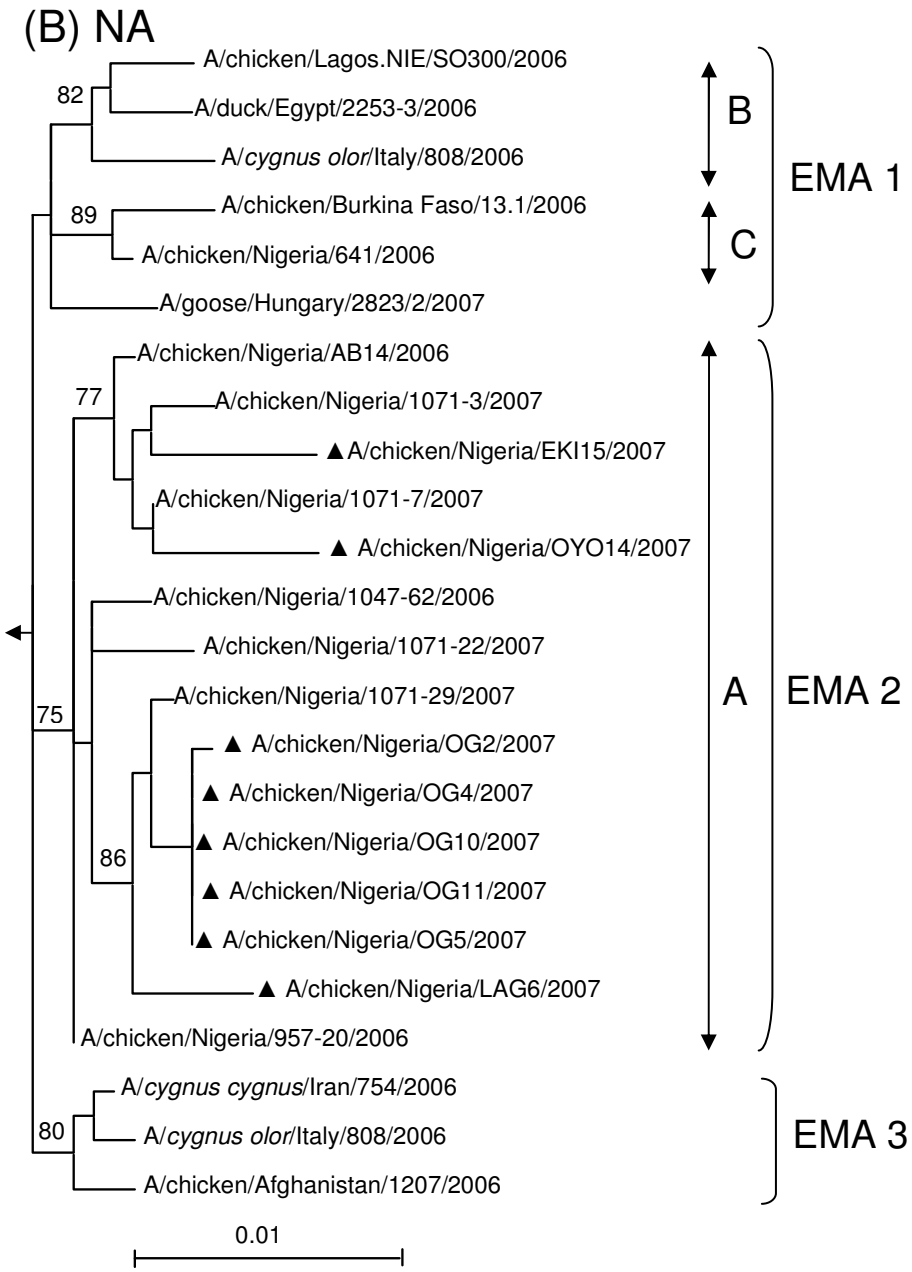


Figure 12. Phylogeny of (A) hemagglutinin (HA) and (B) the neuraminidase (NA) gene from eight HPAI (H5N1) viruses collected in Nigeria during the second half of 2007 (labelled with ▲), in comparison with previously identified sublineage A (EMA 2), sublineage B and C (EMA 1), and EMA 3 strains (Ducatez 2006; Salzberg 2007).

The tree was calculated by Maximum Likelihood method implemented in PAUP 4.0 (Wilgenbusch 2003). The substitution model was obtained by using MODELTEST (Posada 1998). Bootstrap values (%) were calculated by Maximum Likelihood method with 1,000 replications and are indicated on key nodes. Scale bars represent ≈1% of nucleotide changes between close relatives. A/duck/Anyang/AVL-1/2001 was used as an outgroup.

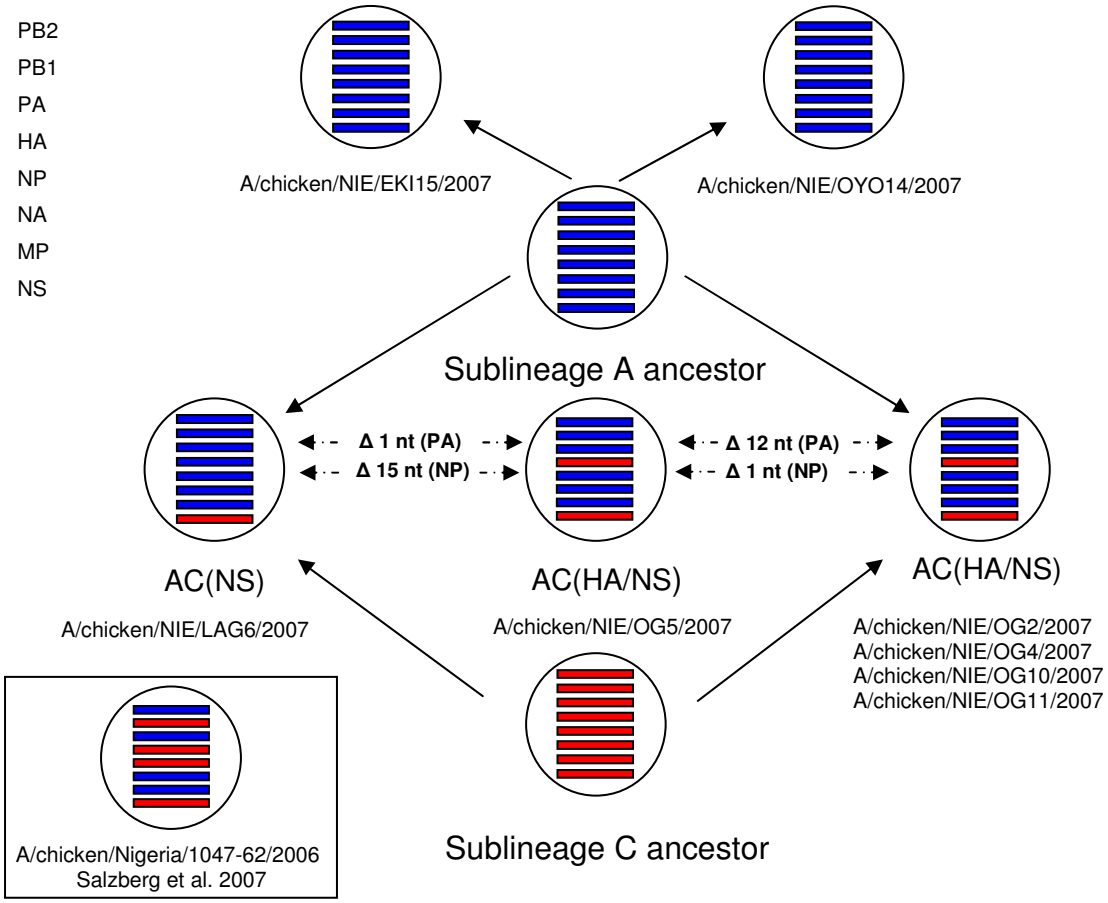


Figure 13. Schematic presentation of sublineage A–derived highly pathogenic avian influenza viruses (H5N1) and reassortants of sublineage A– and sublineage C–derived viruses identified in Nigeria in 2007.

The reassortant reported from Salzberg and others in 2007 (Salzberg et al. 2006) is also shown. Sublineage A–derived gene segments are shown in blue; sublineage C–derived gene segments are shown in red. Gene segments are represented in the order indicated in the right upper corner.

2.3. Mutations

The amino acid sequences of the HA cleavage site (PQGERRRKKRG) of the strains described here are identical to those of all HPAI (H5N1) strains reported from West Africa. All viruses had identical aa in all positions of the HA protein that are associated with preferential binding to α2,3-linked sialic acid (Ha 2001; Shinya 2004) as described (Ducatez 2007a). As for all HPAI (H5N1) strains from Africa, the above viruses had the virulence marker lysine (K) in position 627 of PB2 associated with accelerated viral replication, reduced host defense, higher mortality rate in mice (Chen 2006), and a wider host range of subtype H5N1 strains (Subbarao 1993b). None of the known markers in the

M2 gene associated with resistance to amantadine (Scholtissek 1998) and in the NA gene associated with resistance to oseltamivir (H274Y) (de Jong 2005) were detected.

3. Discussion

Gene sequences of all eight HPAI viruses (H5N1) described here were more closely related to sublineages A or C strains found in Nigeria than to any other published H5N1 virus subtypes. In particular, they were more closely related to the first strains found in Nigeria in the beginning of 2006 than to any strains found outside the country. Thus, the viruses detected in southwestern Nigeria during the second half of 2007 probably evolved from the first viruses brought into the country in early 2006 (Ducatez 2006), suggesting that HPAI (H5N1) has continuously circulated and is endemic to Nigeria. Sublineage A viruses have continued to circulate in Nigeria, whereas sublineage B was found only once on a single farm (SO layer farm, Lagos, January 2006), and sublineage C viruses were no longer detected in 2007. Sublineage A viruses have been detected in northeastern Nigeria in February 2007 (Monne 2008) and in two states of southwestern Nigeria during the last quarter of 2007 (A/chicken/NIE/EKI15/2007 and A/chicken/NIE/OYO14/2007). Sublineages B and C viruses may have been eliminated in Nigeria by effective countermeasures.

All $AC_{HA/NS}$ described here were obtained from chicken flocks in Ogun State from June through August 2007. These results are similar to those found in the beginning of 2007 in other states of Nigeria (Monne 2008). In addition, an AC_{NS} reassortant in Lagos State (A/chicken/NIE/LAG6/2007) distinct from the latter strain was identified. At least two separate reassortment events were necessary to generate sublineages A and C reassortants $AC_{HA/NS}$ and AC_{NS}, which probably had occurred already in 2006, as suggested by the conspicuous absence of sublineage C in 2007. Although it is obviously more difficult to demonstrate reassortment events between genetically similar viruses, the asymmetry in gene divergence of A/chicken/NIE/OG5/2007 compared with the other $AC_{HA/NS}$ and AC_{NS} reassortants suggests that additional reassortment events have taken place.

In 2006, only one reassorted strain was found among 35 European–Middle Eastern–African strains, including 19 viruses reported from Nigeria, belonging to three parent sublineages (Ducatez 2006; Ducatez 2007a; Ducatez 2007b; Salzberg 2007). In the

beginning of 2007, ten of 12 from northern, southern, and central states all belonged to the same $AC_{HA/NS}$ reassortants (Monne 2008), distinct from the $AC_{PB1/HA/NP/NS}$ reassortant detected in 2006 (Salzberg 2007). Similar reassortants were also found in other regions of sub-Saharan Africa (unpublished data). During the second half of 2007, six reassortants including three distinct reassortants among eight strains collected from eight farms located in four contiguous Federal States of Nigeria were identified (Figure 13). These results suggest that reassortants have largely replaced the initial sublineages from which they were derived and that reassortments are pervasive. This finding confirms that reassortments between subtype H5N1 viruses occur frequently when different strains cocirculate in the same region (Macken 2006) and is of particular concern if the increasing prevalence is the result of adaptation to the African environment.

Although segments of the replication complex (PB1, PB2, PA, and NP) may reassort individually without affecting viral fitness (Macken 2006), there seems to be a coordinated evolution of the HA and NA genes (Wagner 2002). In all but one of the Nigerian reassortants, HA and NA genes originated from different sublineages (C and A), suggesting compatibility between phenotypes of both sublineages. All reassortants from Nigeria included sublineage C–derived NS genes, which may suggest a higher fitness of these viruses. Sublineage C–derived NS1 and NEP/NS2 sequences from all Nigerian reassortants and 11 unpublished sequences from $AC_{HA/NS}$ reassortants identified in other sub-Saharan regions showed two specific aa (NS1 V194 and NEP/NS2 R34), which were never identified in sublineage A viruses. It has been shown that modifications in the NS proteins, including aa adjacent to V194, may modulate the virulence of HPAI (H5N1) (Twu 2006; Zhu 2008). Alternatively, the observation that all reassortants in West Africa have sublineage C–derived NS genes may suggest a better adaptation to the African environment of viruses that came from the cold temperatures of central Asia. Thus, the influence of differences in ecology between Africa and Eurasia on viral selection and dynamics deserves further attention.

Although no reassortments have been reported among clade 2.2 viruses (www.who. int/csr/disease/influenza/tree_large.pdf) in Central Asia, Europe, and the Middle East since their emergence from Qinghai Lake region in 2005, reassortments of these viruses seem to be rampant in sub-Saharan Africa, where they have become the critical

determinant of genetic diversity of HPAI (H5N1). Because of low prevalence, mainly in wild birds, clade 2.2 viruses have few opportunities to reassort in Eurasia. In contrast, opportunities to reassort seem to be frequent in sub-Saharan Africa because of great difficulties in setting up a sensitive surveillance system in a complex socioeconomic environment, where backyard farms and large commercial farms with variable biosafety levels coexist, and where culling may threaten the livelihood and survival of the farm.

If the high prevalence of reassortants was typical for West Africa in 2007, the absence of such reassortants anywhere else suggests that reintroductions of subtype H5N1 from Western Africa into Eurasia must be rare. Moreover, all HPAI (H5N1) strains from Nigeria in 2007 were more similar to those found in Nigeria in 2006 than to even the closest relative from Europe in 2007 (Hungary). Although subtype H5N1 has been found in wild birds from Africa, such as vultures (Ducatez 2007b), HPAI (H5N1) has so far not been reported in long-distance migrating birds in West Africa. Thus, the exchange of subtype H5N1 between Eurasia and Africa seems to be a rare event, which in 2006 may have been triggered by unusual bird migration as a result of the central Asian cold spell.

The biological significance of reassortments between genetically similar viruses may be arguable, but the frequency of reassortment events is an important marker of virus endemicity in a region. Moreover, endemicity of HPAI (H5N1) and a high propensity of reassorting in a region where seasonal influenza is unchecked are essential ingredients of the anticipated pandemic.

Part 2

Results of part 2 were published as:

Mossong J, Opp M, Gerloff N, et al. Emergence of oseltamivir-resistant influenza A H1N1 during the 2007-2008 winter season in Luxembourg: Clinical characteristics and epidemiology (2009) Antiviral Res. 84:91–94

Emergence of oseltamivir-resistant influenza A H1N1 during the 2007-2008 winter season in Luxembourg: Clinical characteristics and epidemiology

During the winter of 2007-2008, a high proportion of oseltamivir-resistant isolates of influenza A subtype H1N1 were observed initially in Europe and spread to other continents during 2008–2009 (Besselaar 2008; Lackenby 2008b; Nicoll 2008; Team 2008; Dharan 2009; Hauge 2009; Meijer 2009). This has caused concerns on the effectiveness of antiviral drugs and the rationale of their national stockpiling as recommended by the WHO and European Center for Disease Control (ECDC) for pandemic planning and preparedness, particular in view of a novel A H1N1 virus likely to cause the next pandemic (Fraser 2009).

A retrospective analysis of all laboratory confirmed influenza cases in Luxembourg during the seasonal epidemic in 2007-2008 was conducted to assess a possible link between the emergence of oseltamivir resistance and prophylactic exposure, treatment or stockpiling of oseltamivir. It was also investigated whether patients with oseltamivir-resistant strains differed in terms of clinical symptoms and epidemiologic characteristics from those with sensitive strains. All statistical tests were conducted using Stata 10.

1. Sample collection and epidemiology

This study was conducted within the framework of the national influenza sentinel surveillance in Luxembourg. During the winter season, a sentinel of 12 general practitioners and four pediatricians sent nose and throat swabs of patients with ILI as well as a weekly summary of clinical data to the National Health Laboratory. In addition, approximately 20% of samples were obtained from non-sentinel doctors (see Table 13).

2. Virus extraction, detection and sequence analysis

Total RNA was extracted directly from clinical specimens either using QIAampMinElute Virus Spin kit (Qiagen Benelux, The Netherlands) or NucliSENS easyMAG (Biomérieux, France). Influenza A and B positive samples were detected using the commercially available Influenza Virus (Flu A/B) primer and probe set (ASRFLU-150N-040) on the

SmartCycler platform (Cepheid, Sunnyvale, California). Resistance to oseltamivir (substitution of histidine by tyrosine H274Y in the NA) in H1N1 strains was determined by pyrosequencing of the corresponding nucleotides in the NA gene (Lackenby 2008a). The 795 terminal nucleotide sequences of NA genes were obtained by cycle sequencing using gene-specific primers, after amplification of the corresponding gene segment by RT-PCR.

3. Phylogenetic analysis

Sequence alignments were performed by using BioEdit (Hall 1999). The phylogenetic tree was calculated with the NJ method (p-distance) using MEGA 4 software (Tamura 2007). The influenza A H1N1 strain A/New Caledonia/20/1999 was used as the outgroup. All sequences from Luxembourg can be retrieved in GenBank under accession numbers FM174405–FM174468. The national data, which are kept up-to-date on a web page (Sentinel Surveillance of Influenza, 2009), were forwarded to the European Influenza Surveillance Scheme on a weekly basis.

4. Results and Discussion

During the winter season (October 1, 2007–May 1, 2008) 1040 patient samples were referred to the Laboratory, of which 270 were positive for influenza A and 198 were positive for influenza B. Of the 270 samples positive for influenza A, 195 (72.2%) samples were characterized as oseltamivir-sensitive H1N1, 59 (21.9%) samples as oseltamivir-resistant H1N1, and seven (2.6%) samples as H3N2 subtypes. The epidemic curve (Figure 15) shows that the emergence of oseltamivir-resistant influenza A H1N1 strains was synchronous with the occurrence of oseltamivir-sensitive influenza A H1N1 strains. The proportion of oseltamivir-resistant influenza A H1N1 strains did not change significantly over the course of the season (non-parametric test on difference of median date, $p = 0.95$), but the influenza B epidemic was delayed by three to five weeks in comparison to the influenza A H1N1 epidemic (non-parametric test on difference of median date, $p < 0.001$). Based on the data routinely collected for all samples, Table 12 shows that the characteristics and symptoms of patients infected with oseltamivir-resistant influenza A H1N1 strains were no different from those infected with oseltamivir-sensitive strains of influenza A H1N1 or influenza B strains.

Table 12 Comparison of demographic characteristics and clinical features of patients with oseltamivir resistant influenza A H1N1, oseltamivir sensitive influenza A H1N1 and influenza B during the 2007-2008 winter season in Luxembourg.

Characteristic	Sample size (% of all influenza patients)	Oseltamivir resistant influenza A H1N1	Oseltamivir sensitive influenza A H1N1	Influenza B	p-value
Mean age in years (SD*)	451 (99.8%)	22.1 (15.5)	22.6 (16.8)	24.8 (18.9)	0.391**
Female	444 (98.2%)	61%	50%	44%	0.057 ***
Mid point of epidemic (median)	452 (100%)	2008/01/31	2008/01/31	2008/02/22	<0.001****
Median maximum temperature (°C)	364 (80.5%)	39	39	39	0.477****
Sudden onset	355 (78.5%)	95%	96%	98%	0.331***
Shivers	355 (78.5%)	67%	58%	63%	0.465***
Headache	355 (78.5%)	76%	79%	84%	0.340***
Muscle pain	355 (78.5%)	74%	64%	74%	0.107***
Fatigue	355 (78.5%)	74%	60%	72%	0.043***
Dry cough	355 (78.5%)	89%	81%	87%	0.251***
Sore throat	355 (78.5%)	70%	58%	66%	0.202***
Runny nose	355 (78.5%)	65%	72%	76%	0.320***
GP member of sentinel	462 (100%)	80%	72%	81%	0.118***

* SD- standard deviation
** one way analysis of variance, F-test
*** chi-square test on two-way table
**** non-parametric test of equality of medians

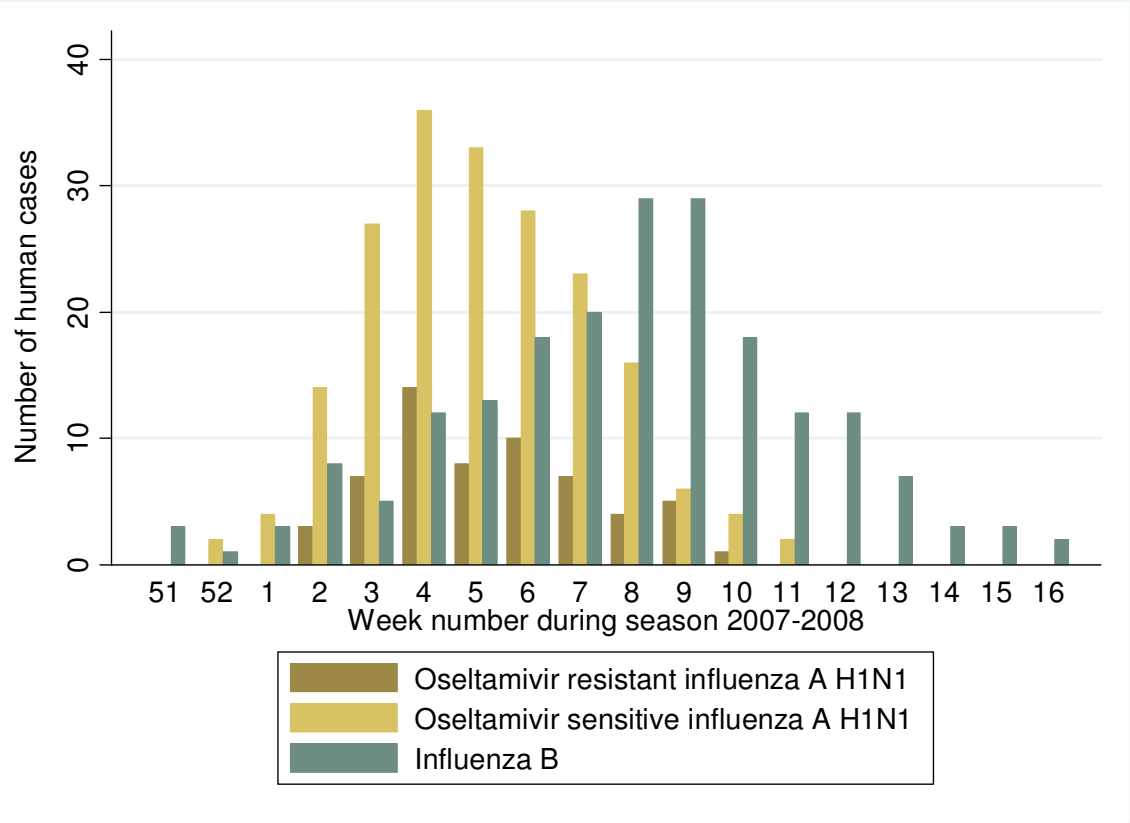


Figure 14: Epidemic curve of influenza in Luxembourg during the 2007-2008 season.

In addition, a rapid retrospective mail-based survey was conducted to collect further information on use and stockpiling of oseltamivir, duration of illness, hospitalization, and attack rates within a household. All 282 patients with laboratory-confirmed influenza reported before the month of March were invited to complete a one-page paper questionnaire. A total of 212 (75%) patients responded. Results shown in Table 13 indicate no significant difference between patients infected with oseltamivir-resistant and oseltamivir-sensitive influenza A H1N1 or with influenza B strains for any of the variables of interest. While a sizable fraction (11.4%) of all patients reported treatment with oseltamivir during acute illness, only one patient (0.4%) reported having stockpiled oseltamivir prior to the present influenza episode and one (0.4%) patient with oseltamivir-sensitive influenza H1N1 reported prophylactic use of oseltamivir. Prior vaccination in our study population was very rare: three patients with oseltamivir-sensitive and two patients with influenza B had been vaccinated (Table 13). Surprisingly, only a minority of patients were in favor of future vaccination, although this must be balanced against the fact that a large proportion of patients were children, and vaccination in children is

currently not recommended in Luxembourg. Attack rates in households were similar between oseltamivir-resistant and oseltamivir-sensitive influenza H1N1 strains and influenza B.

Table 13: Clinical and epidemiologic characteristics of survey respondents.

Patient characteristics	Oseltamivir resistant influenza A H1N1	Oseltamivir sensitive influenza A H1N1	Influenza B	p-value
Sample size	42	112	57	
Mean age in years (SD*)	22.2 (15.6)	20.5 (16.3)	8.2 (21.2)	0.858**
Female	64%	54%	42%	0.088****
Median duration of illness in days	7	6	8	0.058***
Median duration of sickness leave from work or school	3.5	4	5	0.255***
Hospitalised	0 (0%)	1 (0.9%)	2 (3.5%)	0.282****
Vaccinated	0	3 (2.7%)	2 (3.5%)	0.71****
Treated with oseltamivir during illness	6 (14.3%)	12 (10.7%)	6 (10.5%)	0.806****
Prophylactic use of oseltamivir	0 (0%)	1 (0.9%)	0 (0%)	1.0****
Stock of oseltamivir at home before illness	1 (2.4%)	0 (0%)	0 (0%)	0.199****
Would accept vaccination in future	15 (35.7%)	26 (23.2%)	18 (31.6%)	0.237****
Attack rate in household (nr of persons ill during same period/nr of persons living in household)	59/149 (39.6%)	141/349 (40.9%)	85/195 (43.6%)	0.702****

* SD- standard deviation
** one way analysis of variance F-test
*** non-parametric test of equality of medians
**** Exact chi-square on two-way table

Figure 15. Phylogeny of partial NA gene sequences (795 nucleotides encoding the NA protein C-terminus) of a representative sample of influenza A H1N1 strains collected during the 2007-2008 seasonal epidemic in Luxembourg and other countries. Sequences from Luxembourg are in bold format and numbers of strains with identical sequences are given in parentheses. Oseltamivir-resistant strains are highlighted (●).

Figure 15 shows that partial NA gene sequences of oseltamivir resistant strains differed by a minimum of two nucleotides from those of sensitive strains. One of the latter had the H275Y amino acid exchange, which induces the resistant phenotype. The second mutation is non-silent (D354G), and this same mutation was also found on the majority of oseltamivir-resistant strains emerging worldwide in 2007-2008 (Meijer 2009). NA gene sequences from oseltamivir-resistant strains circulating in Luxembourg did not form a separate cluster as compared with resistant strains from other countries. Thus, the unexpectedly high frequency of oseltamivir-resistant influenza A H1N1 in Luxembourg was most probably correlated with the widespread circulation of such viruses in Europe.

From the data of Laboratory confirmed influenza outpatients from this study, little evidence was found that newly emerging oseltamivir resistant influenza A H1N1 strains in 2007-2008 were any different from other circulating influenza strains in terms of patient characteristics, clinical picture, or epidemiology. Following initial seeding in Luxembourg at the start of the season, oseltamivir-resistant strains appear to have spread at a similar rate as oseltamivir sensitive strains, i.e. antiviral drug resistance did not seem to affect fitness. Thus, the results concur to a large extent with previous reports from different parts of the world [Europe (Hauge 2009; Meijer 2009), South Africa (Besselaar 2008) and the United States (Dharan 2009)]. However, the determinants of the relative frequency of oseltamivir resistance occurring in other European countries remain unclear and intriguing. Finally, the data presented with this study will provide an invaluable baseline for the assessing the severity of the pandemic novel A H1N1 strain spreading across the globe (Fraser 2009; Lipsitch 2009).

Part 3

Results of part 3 were published as:

Gerloff NA, Kremer JR, Mossong J, Opp M, Muller CP. Genomic diversity of oseltamivir-resistant influenza virus A (H1N1), Luxembourg, 2007–08. Emerg Infect Dis. 2009 Sep; Vol. 15, No. 9

Genomic diversity of oseltamivir-resistant influenza virus A (H1N1), Luxembourg, 2007-2008

The prevalence of oseltamivir-resistant influenza viruses A (H1N1) (ORVs) increased dramatically worldwide during the winter of 2007-2008 (2008a). Recent reports indicated that by early 2009 most seasonal influenza virus (H1N1) strains were resistant to oseltamivir (WHO 2009b). Resistant viruses were transmitted readily and were as viable and pathogenic as oseltamivir-sensitive viruses (OSVs) (Rameix-Welti 2008; Meijer 2009). The histidine-to-tyrosine (His275Tyr, N1 numbering) mutation in the NA genes of influenza virus A (H1N1) that confers resistance to oseltamivir has previously been associated with impaired virus replication, infectivity, and pathogenicity (McKimm-Breschkin 2003; Yen 2005).

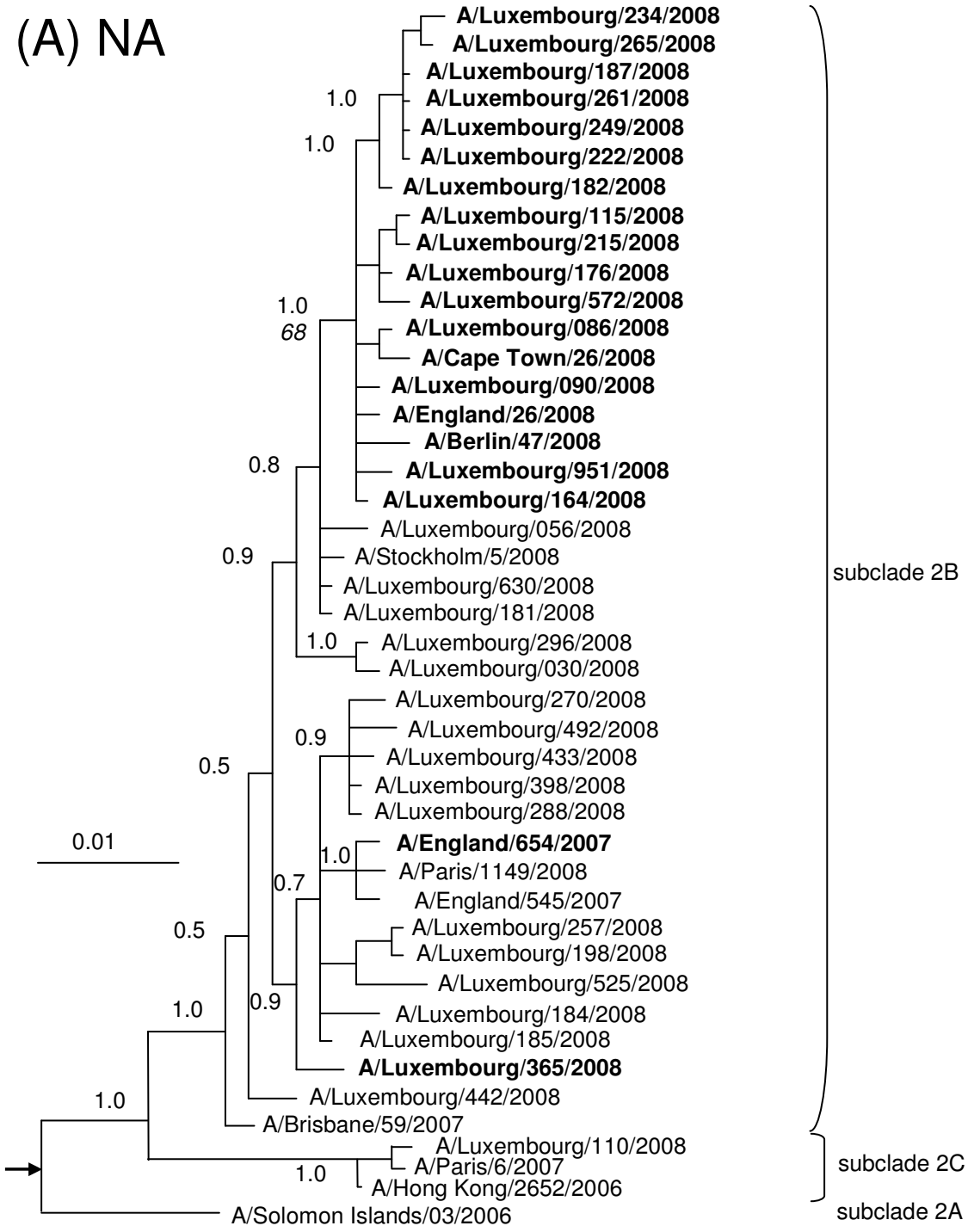
1. Sample collection, gene amplification and sequence analysis

In this work the genetic diversity in all eight gene segments of representative ORVs and OSVs was investigated collected during December 2007–March 2008 by the National Influenza Sentinel Surveillance System in Luxembourg (www.lns.public .lu/statistiques/grippe). Phylogenetic analyses were performed in MEGA version 4, a NJ tree with 10,000 replicates was generated to calculate bootstrap values, shown on the node dividing resistant and sensitive strains (Tamura 2007). Tree topology and posterior probabilities were calculated by using MrBayes version 3 (Ronquist 2003). As the best-approximating model of nucleotide evolution, the general time reversible model with a gamma rate distribution was chosen for the Bayesian analysis. Markov chain Monte Carlo sampling was implemented in MrBayes (Ronquist 2003). In all cases, six chains with at least four million generations were calculated (10% burn-in removed). At least two independent runs of each analysis were performed. Posterior probabilities of the consensus tree topologies were estimated by sampling likelihood parameters every 125 generations. The sequences have been submitted to GenBank (accession nos. FM174406–60, FN401430–45, and FN401487–FN401518).

2. Results and Discussion

Among 140 viruses, 34 strains (24.3%) had the oseltamivir-resistant genotype (Tyr275) in the NA gene. Bayesian analyses of NA genes showed that ORVs formed a distinct cluster supported by high posterior probability (1.00) on the common node (Figure 16). One resistant strain (A/Luxembourg/365/2008) was more closely related to OSVs (minimal Kimura distance 0.3%, 4 nucleotides) than to ORVs (minimal Kimura distance 0.5%, 6 nucleotides). In NA protein, 33 ORVs showed the common Asp354Gly substitution in addition to the Tyr275 mutation. The resistant outlier LNS-365 encoded Asp354 like all other OSVs (n = 106). Similarly, only four other resistant strains from Europe from the same season shared Asp354 with all 2007–2008 sensitive influenza virus (H1N1) strains (n = 251) available in public databases.

A total of 18–44 selected sequences from each of the other genes of ORVs and OSVs were generated to investigate which other genetic markers cosegregated with the resistant genotype. Sequences derived from most of the other genes (PB1, PA, HA, NP, MP, NS) of ORVs and OSVs were phylogenetically interspersed with no distinct clustering. In contrast, matching the phylogeny of NA, PB2 sequences of genotypically resistant strains (n = 14) formed a distinct cluster supported by high posterior probabilities (1.00) and separate from all OSVs (n = 16) and the resistant outlier A/Luxembourg/365/2008 (Figure 16). On the PB2 amino acid level, all OSVs and the resistant outlier A/Luxembourg/365/2008 shared Pro453, whereas all ORV encoded serine at the same position (Ser453). The outlier A/Luxembourg/365/2008 differed only by two amino acids from OSVs but by four amino acids from the closest resistant strain.


(A) NA
A/Luxembourg/234/2008
A/Luxembourg/265/2008
A/Luxembourg/187/2008
1.0
A/Luxembourg/261/2008
A/Luxembourg/249/2008
1.0
A/Luxembourg/222/2008
A/Luxembourg/182/2008
A/Luxembourg/115/2008
A/Luxembourg/215/2008
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68
A/Luxembourg/086/2008
A/Cape Town/26/2008
A/Luxembourg/090/2008
A/England/26/2008
A/Berlin/47/2008
0.8
A/Luxembourg/951/2008
A/Luxembourg/164/2008
A/Luxembourg/056/2008
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0.9
A/Luxembourg/492/2008
A/Luxembourg/433/2008
0.5
A/Luxembourg/398/2008
A/Luxembourg/288/2008
0.01
A/England/654/2007
1.0
A/Paris/1149/2008
0.7
A/England/545/2007
A/Luxembourg/257/2008
0.5
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1.0
0.9
A/Luxembourg/184/2008
A/Luxembourg/185/2008
A/Luxembourg/365/2008
1.0
A/Luxembourg/442/2008
A/Brisbane/59/2007
A/Luxembourg/110/2008
A/Paris/6/2007
1.0
A/Hong Kong/2652/2006
A/Solomon Islands/03/2006
subclade 2B
subclade 2C
subclade 2A

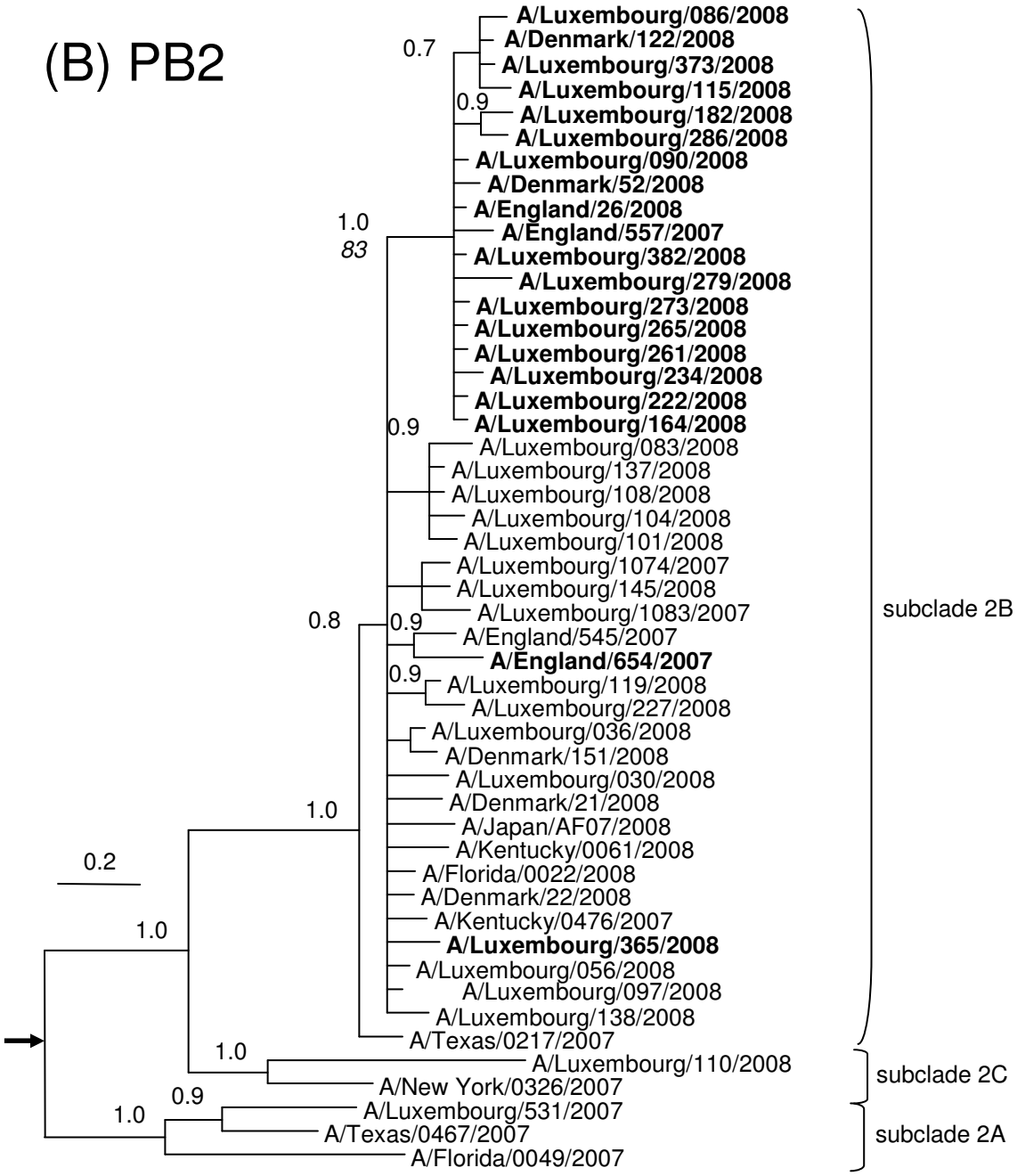

Figure 16. Phylogeny of (A) NA gene (complete gene) and (B) PB2 gene (C-terminal 1,300 nt) for selected influenza viruses A (H1N1) from Luxembourg and other countries.

Subclades are identified at the right of each tree. Posterior probabilities are indicated on important nodes of the consensus tree topologies. Boldface indicates sequences of oseltamivir-resistant influenza viruses A (H1N1) with the Tyr275 mutation in NA. Bootstrap values are shown in italics on the node dividing resistant and sensitive strains. Scale bars indicate nucleotide substitutions per site. The trees are rooted on A/New Caledonia/01/1999 and A/BrevigMission/1918 (indicated by an arrow).

All published PB2 sequences for influenza virus (H1N1) strains collected since 1918 (n = 720) encoded either Pro453 or His453. Until the emergence of ORVs in 2007, Ser453 was only present in three other strains (A/Wilson-Smith/1933 and two strains from 1976 and 1988). Located on the surface of the PB2 cap-binding domain (Guilligay 2008), the Pro453Ser mutation may influence polymerase function and virus replication. The fact that PB2 sequences of ORVs and OSVs are phylogenetically segregated suggests a link between the genetic background and the unexpected fitness of ORVs. There was no amino acid mutation in any of the other genes that segregated in the same way between ORVs and OSVs other than Ser453 (PB2).

Only a single OSV strain from Luxembourg in 2007–08 (A/Luxembourg/110/2008) was derived from subclade 2C, unlike the other 139 influenza virus (H1N1) strains (subclade 2B, Figure 16). Like many other subclade 2C strains, which were recently identified, this virus encoded the amantadine-resistance marker Asn31 in the matrix 2 protein (Niman 2009). Although no reassortments between ORVs and OSVs were identified, double-resistant strains may result from cocirculation of amantadine-resistant strains and ORVs in the same region.

The phylogeny of ORVs identified worldwide (WHO 2009b) indicates multiclonal emergence of resistance, which suggests that OSVs may contain low levels of ORV subpopulations. Using pyrosequencing, the incidence and level of mixed alleles in codon 275 of the NA gene (CAT, sensitive and TAT, resistant) was determined. In 98 clinical specimens (78 sensitive and 20 resistant strains) no minority alleles were reliably detected above the 3% threshold of the assay. Six OSVs with values between 2.1% and 2.9% were further analyzed by cloning of partial NA genes. No evidence of ORVs (Tyr275) was found in NA sequences of 227 clones.

In summary, amino acid markers in NA (Gly354) and PB2 (Ser453) proteins were described, which were present in ORVs but absent in all OSVs from Luxembourg in 2007– 2008. ORVs without this background did not spread as efficiently and were rarely found in Europe. At least one resistant virus was more similar to OSV, which suggests more than two clones of resistant viruses in Luxembourg, potentially with different viral

fitness. We speculate that a new genetic background that is most likely encoded in the PB2 gene may cause the unexpected fitness of the 2007–2008 influenza viruses (H1N1).

Part 4

Results of part 4 were submitted to Emerg Infec Dis:

Gerloff NA, Kremer JR, Charpentier E, Weicherding P, Schuh J, Van Reeth K, Muller CP. (2010) Neutralizing antibodies against pandemic (H1N1) 2009 and avian-like H1N1 swine influenza virus in swine contacts, Western-Europe

Neutralizing antibodies against pandemic (H1N1) 2009 and avian-like H1N1 swine influenza virus in swine contacts, Western-Europe

In April 2009, a new H1N1 influenza A virus emerged in Mexico (CDC 2009b; 2009d). Within weeks, the virus spread first to the US, before becoming the first pandemic influenza virus of the 21st century. Phylogenetic studies revealed that the pandemic A/H1N1 was the result of genetic reassortments between at least two existing swine influenza viruses (SIVs) (Dawood 2009). The HA gene of this novel H1N1 virus is similar to that of "classical swine" and triple reassortant H1N1 viruses that are endemic in swine populations in North America. Remarkably, the pandemic A/H1N1 virus had never been detected in swine populations anywhere at the time of its occurrence in humans, but it is supposed to have circulated undetected in regions with no or minimal surveillance for influenza viruses in pigs.

The virus was most likely absent in swine in Western Europe, as by the "European Surveillance Network for Influenza in Pigs" (www.esnip.ugent.be), which operated from 2001 to 2008 has never been reported it. At this time of writing, there have been reports of pandemic A/H1N1 infection in pigs in Norway (Hofshagen 2009), and sporadic cases in a few other countries on the European mainland, including Germany, Italy and Denmark (WAHID 2009). The pigs seem to have been infected by infected humans, whereas transmission from pigs to humans has not been documented so far. The pandemic A/H1N1 virus is the first swine-origin virus that has the ability to transmit readily between humans (Smith 2009b).

Human infections with SIV have been rare: only 50 cases of zoonotic infections have been reported between 1958 and 2005, mostly in contacts with swine (Myers 2007). Limited secondary transmission to close contacts has been reported but appears to be rare, and sustained human-to-human transmission of enzootic SIVs has never been observed (Shinde 2009). Some serological studies suggest that swine workers are at increased risk of zoonotic infection with SIV (Gregory 2003; Ayora-Talavera 2005; Ramirez 2006; Gray 2007; Robinson 2007; Newman 2008).

The predominant H1N1 SIVs in Europe have an entirely avian genome and were introduced from wild ducks to pigs in 1979 (Pensaert 1981; Brockwell-Staats 2009; Van Reeth 2009b). These viruses are designated as "avian-like" H1N1 and they are antigenically distinct from the H1N1 SIVs in North America, and thus also from the pandemic A/H1N1 virus. There are only a few rare reports of human infection with these avian-like swine H1N1 viruses, and chains of transmission have not been observed (Gregory 2003; Myers 2007; Van Reeth 2009b). There are also no serological studies that would provide indirect evidence of transmission of SIVs to humans in Europe (Van Reeth 2009b).

In studies in the US, UK and Finland antibodies reacting with pandemic A/H1N1 were found in particular in the elderly (CDC 2009c; Ikonen 2010; Miller 2010). This can be explained by the antigenic evolution of the seasonal human H1N1 viruses, which are derived from the 1918 pandemic virus like the classical swine H1N1 virus but have undergone greater antigenic drift than the swine virus (Gatherer 2009). Thus the H1N1 viruses circulating in humans before the 1950s are likely to be more closely related antigenically to the classical swine virus and thus to the pandemic A/H1N1 virus than contemporary human H1N1 viruses.

Here it was investigated whether professional swine contacts have neutralizing antibodies against the 2009 pandemic H1N1 virus and the European avian-like H1N1 SIV. In this study it is shown that swine workers have more often and higher titers of antibodies against pandemic flu and SIV than the control population.

1. Study population

Between July 20 and 28, 2009, sera were collected after informed consent was obtained, from 211 healthy individuals with past or present professional contact with swine. All participants completed a questionnaire about the nature of their contact with swine (occupation, duration, frequency), and influenza vaccination and infection history. None of the participants reported an infection with pandemic influenza A/H1N1. A total of 224 sex and age matched control sera of the general population were obtained from the serum bank of the Laboratoires Reunis, Junglinster in Luxembourg. The samples had been submitted in December 2008 for routine serological testing from residents of

Luxembourg. Because of ethical constraints, no further information was gathered from the controls. The study was approved by the National Ethical Committee for Research in humans.

2. Virus neutralization assay

Sera were tested according to recommended WHO protocols (WHO 2005) by virus neutralization (VN) assay against an influenza A (H1N1) strain isolated from a patient in Luxembourg in July 2009 (A/Luxembourg/43/2009). Complete genome analyses revealed that the sequence was almost identical to the prototype vaccine virus (A/California/7/2009) and represented a typical North American/European pandemic A/H1N1 virus (Smith 2009b). Nucleotide sequences are available on GenBank (accession nos. FN423708-15). The influenza strain A/swine/Belgium/1/98 is representative of the avian-like H1N1 SIVs that are enzootic in swine populations of Western Europe (Van Reeth 2009a). Both viruses have an antigenically distinct H1 with only about 72% homology in the HA1 region at the aa level (Kyriakis 2010). The viruses were grown in MDCK cells.

Positive control sera were collected from five patients no less than five weeks after recovery from a laboratory confirmed infection with pandemic A/H1N1 and from an influenza naïve pig four weeks after experimental infection with A/swine/Belgium/1/98 (H1N1) (Van Reeth 2009a). Prior to the VN assay, all sera were heat-inactivated for 30 minutes at 56°C to inactivate complement and unspecific inhibitors. Titers were reported as the reciprocal of the highest dilution of serum that neutralized completely viral growth. Sera were first screened with a 1:10 dilution at least in duplicates. All sera that showed virus neutralization in at least one well were further titrated in quadruplicates up to a dilution of at least 1:320. The CPE was read after three days. Positive control sera against both viruses were included in all VN assays.

3. Statistical methods

Geometric mean titers (GMT) were calculated for each individual from quadruplicates. All negative sera were given an arbitrary GMT value of five. GMTs of the different groups were compared with the non-parametric Wilcoxon rank sum test. To examine bivariate risk factors associated with antibody prevalence, GMTs of all positive sera were

dichotomized for different cut-off points (≥10 to ≥80) and analyzed by χ^2-test and for low proportions by *z*-test. The distribution of antibody levels was checked for associations with multiple risk factors by using proportional odds modeling (McCullagh 1980; Capuano 2007). Statistical analyses were performed in SigmaStat v3.1.

4. Results

4.1. Study cohorts

The mean age of 211 swine contacts (SwC) was 48.2 years (range 18-94 years); 67.8% were male (Table 14). Most SwC reported having worked on a daily basis (84.8%) in close contact with swine (distance <1m, 83%) for at least ten years (73.5%). One hundred thirty-three SwC were farmers involved in pig breeding, fattening or general farming with pigs and 51 were slaughterhouse workers. Twelve veterinarians, 13 butchers, and two hunters with frequent contact to living swine and/or swine products were also included as SwC. The 224 persons of the age and sex matched control group (controls) had a mean age of 47.6 years (range 18-94 years) and 67.4% were male.

Table 14. Characteristics of study participants at enrollment

	Study sample, no. (%)	
Variables	Swine contacts (n= 211)*	Controls (n = 224)†
Sex		
Male	143 (67.8)	151 (67.4)
Female	68 (32.2)	73 (32.6)
Age group (y)		
18-40	69 (32.7)	80 (35.7)
41-50	59 (28)	58 (25.9)
51-60	39 (18.5)	41 (18.3)
61-94	44 (20.9)	45 (20.1)
Mean [median] age (y)	48.2 [48]	47.6 [47.2]
Years worked in swine production		
<1	4 (1.9)	-
1-4	26 (12.3)	-
5-10	26 (12.3)	-
>10	155 (73.5)	-
Missing	0	224
Frequency of swine contact		
Rarely	3 (1.4)	-
Monthly	2 (0.9)	-
Weekly	25 (11.8)	-
Daily	179 (84.8)	-
Missing	2 (0.9)	-
Frequency of close contact with swine (<1m distant)		
Never	1 (0.5)	-
Rarely	3 (1.4)	-
Occasionally	10 (4.7)	-
Often	22 (10.4)	-
Always	175 (82.9)	-
Self-reported influenza vaccine in the past (5 years)		
No/unsure	155 (73.5)	-
Yes	56 (26.5)	-
Self-reported infection with seasonal influenza		
No	145 (68.7)	-
Yes	57 (27.0)	-
Year of exposure to pigs		
Only until 1997	26	-
Only until 2007	59	-
Until time of collection*	152	-

*Sampled in July 2009
†Sampled in December 2008

4.2. Comparison of antibodies to pandemic A/H1N1 in SwC and controls

SwC had a significantly higher GMT against pandemic A/H1N1 (8.7; 95% CI 7.5-10) in comparison to controls (6.1; 95% CI 5.6-6.6; p=0.004). In absence of a reliable correlate of protection for neutralization titers, data were further analyzed using different cut-off

values for positivity. Table 15 shows that twice as many SwC had neutralizing antibodies against the pandemic A/H1N1 than the controls (21.8% vs. 10.2%) when the lowest cut-off (≥10) was chosen (p=0.001). This ratio slightly increased with rising cut-off values, and remained significant up to a cut-off of ≥1:160 (Table 15). In all age groups, about twice as many SwC than controls had antibodies against pandemic A/H1N1 (cut-off ≥10), except for persons older than 60 years (8.6% vs. 6.7%; Table 16). GMTs in SwC (5.6, 95% CI 4.5-6.9) and in controls (5.4, 95% CI 4.6-6.4) older than 60 years against pandemic flu were similar (p=0.897). In contrast, in younger (<60 years) SwC, antibody levels against pandemic H1N1 were significantly higher (GMT 7.4; 95% CI 6.6-8.2) than in the younger controls (GMT 5.5; 95% CI 4.8-6.3; p<0.05). In SwC, seroprevalence against pandemic H1N1 tends to decrease with age for all cut-offs and in controls, the same was observed for titer cut-offs from 1:10 to 1:40. GMTs of SwC were significantly higher in younger age groups (9.2, 95% CI 7.6-11.1) as compared with those >60 years of age (5.6, 95% CI 4.5-6.9; p=0.021), but not for the controls (6, 95% CI 5.5-6.7 vs. 5.4, 95% CI 4.6-6.4; p=0.403).

Thus, in particular young contacts had more often higher levels of antibodies against pandemic H1N1 than controls and older SwC. The difference between SwC and controls disappeared in the older age brackets and was weaker when older and younger controls were compared.

4.3. Comparison of antibodies to SIV in swine contacts and controls

Similar to pandemic A/H1N1, GMT against SIV was higher in the SwC (10.3; 95% CI 8.8-12) than in controls (7.7; 95% CI 6.9-8.5) however the difference was not significant (p=0.168). More SwC than controls were positive against SIV irrespective of the cut-off chosen (Table 15). These differences were significant for titer cut-offs between ≥20 and ≥160, and increased with higher cut-offs (Table 15). Comparable to findings for pandemic A/H1N1, in age groups up to 60 years of age, 1.2 to 2 times more SwC had antibodies against SIV than controls (cut-off ≥10; Table 16) and also GMTs in this age bracket were significantly higher in SwC than in controls (9.8, 95% CI 8.1-11.8 vs. 6.4, 95% CI 5.8-7; p=0.028). Individuals from both groups above the age of 60 years had similar seroprevalences and GMTs (Table 16).

In contrast to pandemic H1N1 the highest proportion of seropositives was found in older individuals, both for SwC (>50 years) and controls (>60 years; Table 16). Older (>60 years) controls had also significantly higher GMTs than younger individuals (13.6, 95% CI 9.9-18.5 vs. 6.4, 95% CI 5.8-7; p=<0.001) but GMTs differed little in SwC (11.2, 95% CI 8-15.5 vs. 9.8, 95% CI 8.1-11.8; p=0.293).

Thus, SwC had more often and higher antibodies against SIV than controls. In contrast to pandemic A/H1N1, older controls showed more often and higher titers to SIV than younger controls; older SwC had more often antibodies against SIV but titers were similar.

Table 15. Neutralizing antibody reactivity of swine contacts and controls, by virus and study group, statistical significance

Viruses (strain name)	Study sample, no. (%, 95% Confidence interval)* Swine contacts (n = 211)	Controls (n = 224)	P-value
Pandemic A/H1N1 (A/Luxembourg/43/2009)			
≥10	46 (21.8%, 16.8-27.9)†	23 (10.3%, 6.9-14.9)‡	0.001§
≥20	37 (17.5%,13-23.2)†	16 (7.1%, 4.4-11.3)‡	0.001§
≥40	31 (14.7%, 10.6-20.1)	12 (5.4%, 3.1-9.1)	0.002§
≥80	14 (6.6%, 4-10.8)	4 (1.8%, 0.7-4.5)	0.02§
≥160	6 (2.8%, 1.3-6.06)	0 (0%, 0-1.2)	0.033¶
≥320	5 (2.4%, 1-5.4)	0 (0%, 0-1.2)	(0.061)¶
Avian-like SIV (H1N1) (A/swine/Belgium/1/98)			
≥10	66 (31.3%, 25.4-37.8)	59 (26.3%, 21-32.5)	(0.289)§
≥20	57 (27%, 21.5-33.4)	38 (17%, 12.6-22.4)	0.015§
≥40	39 (18.5%, 13.8-24.3)	12 (5.4%, 3.1-9.1)	<0.001§
≥80	21 (10%, 6.6-14.7)	4 (1.8%, 0.7-4.5)	<0.001§
≥160	9 (4.3%, 2.3-7.9)	1 (0.4%, 0.1-2.5)	0.019¶
≥320	4 (1.9%, 0.7-4.8)	1 (0.4%, 0.1-2.5)	(0.331)¶

*Data are number of individuals with antibodies (%, 95% Confidence interval); p values >0.05 cut-off for significance are shown in parentheses.
†P<0.05 when compared to swine contacts against avian-like SIV (H1N1) of the same titer cut-off.
‡P<0.003 when compared to swine contacts against avian-like SIV (H1N1) of the same titer cut-off.

§χ2 test on two-way table.
¶z-test.

4.4. Comparison of antibodies against pandemic flu and avian-like H1N1 SIV

For all cut-offs, SwC had a higher seroprevalence for SIV than for pandemic virus. Controls had a higher seroprevalence against SIV than pandemic virus but only for the lower titers (≥10, ≥20; Table 15). The differences in positivity between the two viruses increased with age in both SwC and controls (Table 16). Comparing seroprevalences of pandemic A/H1N1 to the SIV, differences were only significant for SwC >60 years (p=0.002). Also significantly more controls of the same age group (>60y) were positive against SIV (62.2%) than against pandemic A/H1N1 (6.7%; p<0.001;Table 16). The proportion of seropositive controls >60 years (62.2%) differed significantly compared to the younger (<60 years, 17.3%; p<0.001) for SIV.

Thus, the comparison of the seroprevalence against both viruses suggests that for both study groups more persons had antibodies against SIV than pandemic flu and that with increasing titers differences in positivity decreased. Mainly older rather than younger participants contributed to positivity against SIV, while positivity to pandemic A/H1N1 resulted mostly from younger individuals.

Table 16. Neutralizing antibody reactivity of equal or higher than minimum detection limit (≥10) of swine contacts and controls, by virus, study group and age

	Pandemic A/H1N1 (A/Luxembourg/43/2009)*		Avian-like SIV (H1N1) (A/swine/Belgium/1/98)*	
Year of birth (age in 2009)	Swine contacts	Controls	Swine contacts	Controls
1969-1991 (≤40 y)	22/69 (31.9%, 22.1-43.6)†	12/80 (15%, 8.8-24.4)	19/69 (27.5%,18.4-39)	15/80 (18.8%, 11.7-28.7)
1959-1968 (41-50 y)	10/59 (16.9%, 9.5-28.5)	5/58 (8.6%, 3.7-18.6)	11/59 (18.6%, 10.7-30.4)	8/58 (13.8%, 7.2-24.9)
1949-1958 (51-60 y)	9/39 (15.3%, 12.7-38.3)	3/41 (7.3%, 2.5-19.4)	17/39 (43.6%, 29.3-59)‡	8/41 (19.5%, 10.2-34)
1915-1948 (≥61 y)	5/44 (8.6%, 5-24)§	3/45 (6.7%, 2.3-17.9)¶	19/44 (43.2%, 29.7-57.8)	28/45 (62.2%, 47.6-74.9)
Total 1991-1915 (18-94 y)	46/211 (21.8% 16.8-27.9)#	23/224 (10.3%, 6.9-14.9)**	66/211 (31.3%, 25.4-37.8)	59/224 (26.3%, 21-32.5)

*Data are no. individuals/total no. individuals in age groups with antibodies at or above ≥10 (%, 95% Confidence interval); P-values <0.05 cut-off for significance were calculated with chi-square test and are indicated behind parenthesis.
†P=0.012, compared to controls of the same age group against the pandemic A/H1N1.
‡P=0.037, compared to controls of the same age group against avian-like SIV (H1N1).
§P=0.002, compared to swine contacts of the same age group against avian-like SIV (H1N1).
¶P<0.001, compared to controls of the same age group against avian-like SIV (H1N1).
#P<0.05, compared to swine contacts against avian-like SIV (H1N1).
**P<0.001, compared controls against avian-like SIV (H1N1).

4.5. Cross reactivity and double positivity

Antibody titers of convalescent sera from pandemic A/H1N1 infected patients were 16 times higher against pandemic flu than against SIV (GMT 226.2 vs. 13.5), indicating a low cross-reactivity between both viruses. Similarly, a pig serum against SIV had a 128 times lower GMT against pandemic flu (>1280 vs. 10).

The percentage of double positives was significantly higher in SwC (13.3%) compared to controls (6.3%; p<0.05). The odds to be double positive were higher for SwC than for controls (odds ratio [OR] 2.3, 95% CI 1.2-4.5, cut-off ≥10) and increased to 3.1 (95% CI 1.4-7.2) for a cut-off of ≥40. A SwC who was positive to SIV, had a 2.4 (OR 95% CI 1.3-4.3) times higher chance to be positive for pandemic flu. For controls that were positive for SIV, the chance was 6 times higher to have a titer against pandemic flu (OR 95% CI 2.9-12.6).

4.6. Risk factors associated with profession, gender or vaccination

SwC had OR of 2.4 (95% CI 1.4-4.2) to 3.9 (95% CI 1.3-12) for antibodies against pandemic A/H1N1 for cut-offs of ≥10 to 80 in comparison to controls. Similarly, for SIV, the OR varied from 1.3 (95% CI 0.8-1.9) to 9.9 (95 % CI 0.5-38.9) for cut-offs of ≥10-160. Male SwC had slightly increased OR of 1.7 (95% CI 0.8-3.5) to be positive against pandemic A/H1N1 and OR of 1.1 (95% CI 0.6-2.3) against SIV compared to female SwC (cut-off ≥10). About one fourth of SwC (26.5%) had self-reportedly received at least one dose of seasonal influenza vaccine during the previous 5 years. For vaccinated SwC the odds slightly increased to have antibodies against pandemic A/H1N1 (OR 1.3 [95% CI 0.6-2.6]) as well as against SIV (OR 1.3 [95% CI 0.7-2.5]; cut-off ≥10) compared to unvaccinated SwC. Swine contacts with pig exposure until the time of sampling had a slightly increased OR of 1.5 (95% CI 0.7-3.3) to have antibodies against pandemic A/H1N1 2009 compared to individuals who had no contact with swine after 2007. SwC who were in contact until time of sampling had an OR of 0.5 (95% CI 0.2-1.1) to have antibodies to SIV compared to individuals who had no contact after 1997. Thus, there were no significant associations between year of exposure and seroprevalence of antibodies against either virus.

5. Discussion

The sera of SwC were collected in late July 2009, about three months after the outbreak of the pandemic strain in Mexico. At that time, the virus had already spread to all continents, but influenza intensity was still low in Europe, in particular in Luxembourg and its neighboring countries. The only countries with increased infection rates were the UK, Ireland and Spain with sporadic outbreaks (ECDC 2005 - 2010). Luxembourg had an intensive active surveillance of ILI. All suspected cases were laboratory confirmed by real-time diagnosis and all confirmed cases were followed up to investigate the origin of the infection at least until beginning of August. Patients and their contacts received prompt antiviral treatment and a home-quarantine was recommended. In Luxembourg, about 60 cases were reported and laboratory-confirmed when the last SwC sera were collected. Until end of June 2009 virtually all of the Luxembourgish patients were epidemiologically unrelated and the source of infection was not determined for only one fifth of the cases (Santé 2007). The first sustained transmissions were observed only by

mid September (pers. comm. J. Mossong). The first cases of pandemic A/H1N1 in swine on the European mainland were only reported in January 2010 (WAHID 2009).

As there is no correlate of protection for neutralizing antibodies or even a definition of a positive titer measured by VN assay (Neuzil 2009) we analyzed the VN titers using running cut-off values for positivity and compared GMTs. This analysis showed a significantly higher prevalence of neutralizing antibodies against the 2009 pandemic virus in SwC than in general population controls and seropositivity decreased with age. Younger SwC (<60 years) had higher titers and twice as many young SwC had neutralizing antibodies against pandemic flu than age matched general population controls. The antibodies to pandemic A/H1N1 in the SwC may be due to serological cross-reactions with other H1 influenza viruses to which SwC have been exposed or to subclinical infections with the pandemic virus during the first months of the pandemic. There is no evidence that the pandemic virus was present in swine in Europe in July 2009 or before. Thus, the true explanation for the higher seroprevalence of antibodies against the pandemic virus in SwC as compared to the control population remains unknown. One limitation of our study is the difference in the timepoints of serum collection between the SwC (July 2009) and the control population (December 2008).

The low levels of neutralizing antibodies against pandemic flu in participants from the general population were in agreement with previous studies (Itoh 2009). We observed lower proportions of positives against the pandemic A/H1N1 strain in the controls older than 60 years of age compared to younger controls, but antibody titers were significantly higher in the older age bracket. Although our findings are in contrast to reports from the UK and Finland (Ikonen 2010; Miller 2010), they are in agreement with two studies from China, where the elderly (≥60 years) had no or a low prevalence of neutralizing antibodies against pandemic flu (Chen 2009; Zhu 2009).

This study further showed a significantly higher prevalence of neutralizing antibodies against the H1N1 SIV in SwC than in the controls at cut-off levels ≥20-160, but GMTs were not significantly different. Similar serological studies in humans in the US showed markedly elevated antibody titers against North American H1N1 and H1N2 SIVs in swine workers as compared to control subjects (Olsen 2002; Myers 2006; Ramirez 2006; Gray

2007; Myers 2007; Newman 2008). These studies used HI instead of VN assays and they report odds ratio's for increased serologic responses instead of seroprevalence rates. The reported odds ratios, however, appear to be higher than those in the present study (Olsen 2002; Myers 2006; Gray 2007). Those higher OR could at least partially be explained by specifically excluding individuals with swine exposure in the US control groups.

Most important, it is uncertain whether the antibodies to SIV in humans correlate with infection, because serologic cross-reactions between human and swine viruses of the H1 subtype cannot be excluded. Most humans undergo sequential infections with multiple antigenic variants of H1N1 and H3N2 human influenza viruses throughout their life. Such sequential infections strongly increase the risk for serologic cross-reactions with antigenically distinct H1 viruses, as documented in experimental studies with pigs (Kyriakis 2010). This may also explain why older individuals in the general population have higher antibody titers to SIV than their younger counterparts. Both older and younger control subjects are unlikely to have been infected with SIV, but older people have been exposed to a wider variety of human seasonal H1N1 viruses. In Luxembourg the elderly in the general population may even have had contact with swine, since between 1920 and 1947 50 to 22% of all Luxemburgish households kept at least 5 pigs, but there was no significant swine influenza activity in this part of Europe before 1979 (Pensaert 1981; STATEC 2010). Apart from antibodies to SIV, part of the control population also had antibodies to pandemic A/H1N1 at a time when the pandemic virus was not yet circulating in humans or in pigs in Europe.

All these findings show that the presence of antibodies to a given influenza virus does not necessarily reflect infection with that virus. It is possible that the elevated antibody titers to SIV in part of the SwC result from exposure to the virus, but further studies are required to determine all possible causes. In any case, neutralizing antibodies should confer at least a partial protection against infection. This may reduce the risk that the avian-like H1N1 SIV would cause significant outbreaks of disease in humans in the near future.

In conclusion, this is the first study measuring neutralizing antibodies against the pandemic A/H1N1 (2009) virus and an avian-like H1N1 SIV in persons with occupational swine contact. We showed that European swine workers have more often neutralizing

antibodies against both H1N1 viruses than the general population. Our study does not allow, however, to estimate the incidence of zoonotic SIV infections and further studies are required to determine to what extent the serological responses correlate with infection.

Chapter V - Conclusions and Perspectives

Influenza A virus' evolution is a complex process characterized by the viruses' constant genomic variation that eventually results in its escape from the host immune response, subsequent reinfection of human hosts, and enhanced pathogenicity. Antigenic and genetic changes shape the viral evolution, causing recurring epidemics and pandemics in humans and outbreaks of HPAI viruses with a high impact on public health and poultry industry.

In Africa, poultry meat serves as one of the major protein sources and Nigeria is one of the largest poultry producers in sub-Saharan Africa. Influenza virus transmission is facilitated by low biosecurity levels in commercial farms located in the southwest of the country. Furthermore, backyard poultry moves without a restriction, which leads to enhanced intermingling of wild and domestic birds (Ducatez 2007b; Bank 2010). In the first study, genetically diverse HPAI (H5N1) from Nigeria were found to originate from various reassortment events, which suggested that, despite large eradication campaigns, HPAI viruses were endemic. In addition to these reassortants identified from outbreaks in late 2007, another study, described the same genotype of reassortant viruses (HA and NS genes from sublineage C) that were found in early 2007 (Monne 2008). This indicated that the reassortment events between sublineage A and C viruses most probably occurred in 2006, and that the reassortant strains spread unhampered, resulting in outbreaks in at least ten different states in Nigeria (Fusaro 2010). Furthermore, the unique composition of the

genome with HA and NS genes from sublineage C and the other six gene segments derived from sublineage A viruses could indicate enhanced fitness of these reassortments, maybe as a result of the African environment. The absence of the same reassortants outside sub-Saharan Africa showed that re-introductions of HPAI viruses from Africa into Eurasia either did not occur or remained undetected. However, in 2008, introduction of new HPAI (H5N1) strains into Nigeria was reported and their phylogeny revealed a close relation to European strains. This constituted the first evidence of a new virus introduction since 2006 into Nigeria (and in West-Africa) and implied migratory birds as the most likely source of transmission (Monne 2008; Fusaro 2010).

The constant risk of avian-to-human transmission is reflected in almost 500 infected individuals as of early 2010 (WHO 2010b). Thus, besides actions directed against the spread of avian influenza in sub-Saharan countries, surveillance programs for human influenza should be improved to hinder the reassortment of human and avian influenza viruses. So far, National Influenza Centers have been established in only a few countries in this region, such as Nigeria, Ghana, and Côte d'Ivoire (WHO 2010d). Their mission is to collect and provide influenza surveillance data to facilitate public health policy enforcement.

In the African environment, Egypt exemplifies the difficulty to set up effective countermeasures against viral spread. The most populated country in Africa has also the highest population densities, which are paired with rearing of backyard poultry in vicinity to houses (Bank 2010). These characteristics facilitate virus transmission from birds to humans as well as within flocks of poultry. Despite vital influenza surveillance in birds, as well as in humans, it is of note that lately all human cases of infection with HPAI (H5N1) in Africa were reported from Egypt, where the virus is considered to be endemic in poultry (OIE 2010; WHO 2010b). This demonstrates that even in a financially well-situated country (in comparison to sub-Saharan countries), containment measures were not able to abolish the viral spread and infections in humans still occur (WHO 2010b). In the future, broader strategies should aim at educational programs to sensitize further people's awareness of HPAI in birds, the risk of transmission to humans and the means of prevention at hand.

Similar to containment strategies against HPAI (H5N1) outbreaks in poultry, epidemiological surveillance of human influenza is a critical factor to control viral spread. Infections caused by seasonal influenza of subtypes H1N1 and H3N2 can be treated with neuraminidase inhibitors, such as oseltamivir. Antiviral resistance against oseltamivir did not occur in natural variants of seasonal H3N2 or H1N1 viruses; however, drug-resistant viruses were described for patients infected with HPAI (H5N1) after prolonged treatment with oseltamivir (de Jong 2005). The mutation conferring resistance towards oseltamivir had previously been associated with impaired virus replication, infectivity, and pathogenicity, nonetheless, during the influenza season 2007 to 2008, resistant H1N1 viruses emerged without selective drug-pressure, and the resistant strains seemed to be natural, spontaneously arising variants that transmitted readily (Moscona 2009).

Our study, conducted together with the National Health Laboratory in Luxembourg, revealed that seasonal drug-sensitive and resistant influenza A H1N1 viruses emerged synchronously after initial seeding. Moreover, epidemiological data suggested that the sudden emergence of drug-resistant H1N1 influenza viruses was not associated with treatment, prophylaxis, or stockpiling of oseltamivir in Luxembourg. Further, the clinical data revealed that the outcome in untreated patients infected with sensitive or resistant strains was similar. From these findings, we suggested that the new drug-resistant variants were as viable as sensitive viruses and that drug-resistant variants transmitted readily in the population.

In the third study, we looked at differences on the genomic level in a subset of drug-sensitive and resistant strains. Comparison of complete and partial sequences of all gene segments identified genetic markers only present in drug-resistant variants but absent in sensitive viruses. Phylogenetic analysis revealed amino acid markers in NA (Gly354) and PB2 (Ser453) proteins which were present in drug-resistant viruses but absent in all drug-sensitive strains from Luxembourg. Thus, oseltamivir-resistant viruses without this genetic background did not spread as efficiently and were rarely found in Europe. At least one resistant virus was more similar to drug-sensitive viruses, which suggests more than two clones of resistant viruses, potentially with different viral fitness. However, neither in pyrosequencing nor in cloning experiments, we were able to identify quasispecies of resistant sequences in sensitive virus strains.

The altered genetic background that is most likely encoded in the PB2 gene may cause the unexpected fitness of the 2007–2008 influenza viruses (H1N1). The PB2, PB1, and PA protein form the replication complex, thus, amino acid mutations could result in enhanced replication rates. This hypothesis can be further investigated by *in vitro* experiments with reassortant influenza viruses generated by reverse genetic systems (Fodor 1999; Neumann 1999). Moreover, site-directed mutagenesis that targets specific nucleotides leading to non-synonymous mutations, can offer another approach to study viral fitness, as amino acid changes alter protein-protein interactions that are involved in the replication process (Gething 1986).

Eventually, oseltamivir-resistant viruses became the prevailing seasonal H1N1 viruses, and replaced oseltamivir-sensitive strains in 2009 (CDC 2009a). It is possible, that drug-resistant influenza viruses acquire new mutations facilitating transmission between diverse species, or turn into double-resistant strains (against both groups of antivirals) through reassortment events (Ducatez 2010). These findings help to assess viral drug-resistance mechanisms and offer new ways to distinguish drug-resistant variants from sensitive viruses that could be applied to H1N1 viruses, like the pandemic strain. However, in the future, it will become vital to develop new treatment strategies against influenza infections that either target viral proteins, less susceptible for escape mutations, or host proteins that are essential for virus replication (Konig 2010).

As major components of humoral immunity, neutralizing antibodies against the antigenic sites on the hemagglutinin protein provide protection against re-infection with influenza viruses. However, this protection is compromised by the constant variation of the viral HA protein that leads to an altered antigenicity and decreased antibody specificity (Francis 1960; Wright 2007). For example, after emergence of the new swine-origin influenza A virus, it was assumed, that the majority of the population would be immunologically naïve to the virus.

Similar to pandemic viruses from 1918, 1957 and 1968, the pandemic strain from 2009 evolved undetected in an intermediate mammalian host for several years before detection in humans (Smith 2009a). Although zoonotic infections with SIV are rare, serological studies suggested that swine worker displayed an increased risk to become infected with

swine influenza viruses, resulting in cross-reactive neutralizing antibodies also reacting with human viruses of subtypes H1N1 and H3N2 (Myers 2006).

We measured neutralizing antibodies against the pandemic A/H1N1 (2009) virus and an avian-like H1N1 SIV in persons with occupational swine contact. Our results showed that European swine contacts have an increased chance of possessing neutralizing antibodies against both H1N1 viruses than the general population, which confirmed findings from earlier studies. In addition, our results suggested that infection with zoonotic influenza viruses in Europe resulted most probably in antibodies able to neutralize SIV. Our study does not allow, however, to estimate the incidence of zoonotic SIV infections and further studies are required to determine to what extent the serological responses correlate with infection. As shown recently, pandemic H1N1 viruses transmitted not only from humans to birds, but also from humans to pigs (WAHID 2009). Multiple introductions of the pandemic virus into swineherds eventually resulted in antigenically diverse reassortants, which depicts the possibility for the virus to evolve into new variants (Vijaykrishna 2010). To strengthen knowledge about SIV and zoonotic infections with SIV, one perspective could be to establish influenza surveillance in pigs combined with close monitoring of influenza infections in high-risk groups, such as individuals in close contact to swine.

The findings of this work advance not only the knowledge about epidemiology and evolutionary mechanisms of influenza viruses in the avian, human, and porcine host but also provide new perspectives in terms of development of drug-susceptibility in natural arising viral variants.

In conclusion, despite the tremendous progress in influenza research, since the virus was isolated for the first time about 80 years ago, the recent pandemic highlighted the continued importance of pandemic preparedness planning, effective seasonal vaccines and potent antivirals. Influenza viruses remain one of the major agents causing respiratory disease to date with a high impact on public health as well as on the poultry industry, however, we are on the right path to fully understand and unravel influenza A virus evolution.

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Annexes

Conference Presentations

Gerloff, NA et al. Genetic diversity of oseltamivir sensitive and resistant strains from Luxembourg (2007/08) at the European Society for Clinical Virology Winter Meeting, January 7-10, 2009, Amsterdam, The Netherlands, published as Gerloff, NA, et al. (2009) O.7.3 Genetic diversity of oseltamivir sensitive and resistant strains from Luxembourg (2007/08). J Clin Virol 44, S15-S15.

Gerloff, NA et al. The spread and evolution of highly pathogenic avian influenza H5N1 in Africa at the 1st BirdFlu conference 2008, Oxford, United Kingdom; published in Conference Proceedings; Birdflu2008: Avian Influenza and Human Health, 10-11 September 2008, St Hilda's College, Oxford, United Kingdom, J Mol Genet Med (2009), 3(1), 182-189

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Acknowledgements

First, I would like to thank my supervisors Prof Claude P. Muller und Prof Nikolaus Müller-Lantzsch to give me the possibility to write this thesis. Prof Muller supported my work in offering advice on both experimental design and scientific writing, which I appreciated a lot. I would like to thank Dr Jacques Kremer who was always available to discuss my ideas and taught me how to look at my results from another perspective. Without his encouragement and constant guidance, I could not have finished this dissertation.

I am deeply grateful for the financial support "Bourse Formation de la Recherche" and "Aides a la Formation de la Recherche" to the Fond National de la Recherche (FNR) of the Ministry of Higher Education of Luxembourg and the Centre de Recherche Public de la Santé (CRP-Santé).

Special thanks go to the numerous international and national collaborators, which were involved in specimen collection, virus identification, and publications, especially Dr Demola Owoade from the University of Ibadan in Nigeria and Dr Mossong from the Microbiology Unit of the National Health Laboratory in Luxembourg. Many thanks also to all visiting scientists and summer students; I had the honor to supervise during my thesis.

From the Institute of Immunology, I would like to thank Dr Mariette Ducatez, who taught me essentially all molecular and phylogenetic techniques and Dr Christophe Olinger, who deepened my knowledge in complex phylogeny. Many thanks go to the best office mates ever, my friends Julia Kessler and Dr Monique Jacoby, for the daily teatime, psychological assistance, and vibrant discussions about experiments, and our feline friends. Special thanks go to the technicians Aurélie Sausy, Émilie Charpentier and Sébastien De Landtsheer for their indispensable work, always available advice and most importantly, delicious chocolate. I am also grateful to Carole Weis for her vital support and of course, I would like to thank all my other former and current colleagues, especially, Andrea, Denis, Dominique, Fred, Judith, Linda, Mario, Nathalie, Oliver, Sara, Simone, Sophie, Stephanie, Tom, Vitor and those who do not fit on this page.

Patrick, villmols Merci fir deng Ennerstëtzung a Frëndschaft.

Ich möchte außerdem Anne und Tina von ganzem Herzen danken für ihre unersetzliche Freundschaft.

Der grösste Dank gilt meiner Familie, die mich bedingungslos unterstützt und immer an mich geglaubt hat. Vielen Dank geht an Mama, Papa, Marco, Svenja, Lennart, Matze, Oma´s Anni und Christa, Opa Robert und alle anderen Familienmitglieder.

Grazie infinite ad Alessandro per il suo supporto anzi la sua toleranza e anche alla sua (la mia) famiglia in Italia. Grazie di cuore a Carmen, Eugenio, Donatella e Claudia, e ai nonni!