# Identification and Characterization of Pathogenicity Determinants of H5N1 Highly Pathogenic Avian Influenza Viruses isolated from the 2015 Poultry Outbreak in Ghana

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## Zusammenfassung

Die Influenza bedingte Morbidität und Mortalität wurde lange Zeit in Ghana vernachlässigt. Erst im Nachgang der H1N1 Pandemie in 2009 sowie nach wiederholten Geflügelpest Ausbrüchen im Land wurden Surveillance Programme gegen humane Influenza initiiert. Ein entsprechendes Programm gegen aviäre Influenza mit dem Ziel das zoonotische Potenzial neuartiger Influenzaviren aus dem Tierreich zu untersuchen ist bis heute nicht existent.
Ziel dieser Arbeit war es, das zoonotische Potenzial von zirkulierenden Influenza A Viren (IAV) in Ghana mittels molekularbiologischer Methoden sowie im Mausmodell zu untersuchen. Dazu wurde zunächst eine Seroprevalenzstudie in Tiermärkten durchgeführt. Die dort arbeitenden Tierhändler waren alle sero-positiv gegen humane 2009 pandemische H1N1 und H3N2 jedoch nicht gegen aviäre H5N1 IAV. Alle untersuchten Tiere in diesen Geflügelmärkten, wie z.B. Geflügel und Schweine waren sero-negativ für die getesteten IAV Subtypen. Diese Daten sprechen dafür, dass in dem untersuchten Tiermarkt keine unmittlebare Tier-zu-Mensch Transmission von IAV stattgefunden hat. Wenige Monate später jedoch wurde über einen H5N1 Geflügelpest Ausbruch auf anderen Tiermärkten berichtet. Dieser Ausbruch unter domestiziertem Geflügel führte zur Keulung von über 100.000 betroffenem Geflügel, das in einem kleinen Land wie Ghana, eine maßgebliche Bedrohung der Nahrungsversorgung bedeutet. Daraufhin wurden im Rahmen dieser Arbeit hochpathogene aviäre Influenza A Viren (HPAIV) des H5N1 Subtyps von infiziertem Geflügel aus den betroffenen Tiermärkten aus insgesamt drei verschiedenen Regionen in Ghana (Accra, Ketu und Obuassi) isoliert und hinsichtlich ihres zoonotischen Potenzials molekularbiologisch und pathologisch untersucht. Die Sequenzanalysen sowie die phylogenetischen Berechnungen der Ghanaischen H5N1 HPAIV zeigten eine hohe Homologie mit 80-99% zu den H5N1 HPAIV Isolaten aus Nigeria, die dort kurz vorher zu einem Ausbruch in betroffenen Tieren führten. Diese neuartigen H5N1 Stämme aus Ghana wurden dem clade 2.3.2.1c zugeordnet, welche nach der Weltgesundheitsorganisation ein hohes zoonotisches Potenzial besitzen. Unsere phylogenetischen Daten sprechen dafür, dass das H5N1 HPAIV wahrscheinlich über den Tierhandel aus Nigeria nach Ghana eingeführt wurde. Weitere molekularbiologische Analysen zeigten, dass alle drei H5N1 HPAIV aus Ghana eine Spezifität gegen aviär-typische Rezeptoren (α2,3-glykodisch gebundene Sialinsäuren) besitzen. Somit scheint noch keine Anpassung des viralen Rezeptor-bindenden Proteins, dem Hämagglutinin, an die human-typischen Rezeptoren (α2,6-glykodisch gebundene Sialinsäuren) stattgefunden zu haben. Untersuchungen zu adaptiven Eigenschaften der viralen Polymerase in humanen Zellen zeigten, dass vor allem das H5N1 HPAIV aus Accra im Vergleich zu einem aviären H5N1 Isolat aus Deutschland (R65) eine erhöhte Polymeraseaktivität besitzt. Diese Aktivität der Accra Polymerase war

jedoch niedriger als die eines humanen H5N1 Isolats aus Thailand (KAN-1). Diese Daten sprechen dafür, dass die H5N1 Polymerase des Accra Isolats bereits teilweise human-adaptive Signaturen erworben hat. Weitere Replikationskinetiken im gesamten Viruskontext in humanen Zellen konnten die erhöhte replikative Fitness des H5N1 Accra Isolats im Vergleich zum H5N1 aviären R65 jedoch nicht zum humanen KAN-1 Isolat bestätigen. Auch diese Daten sprechen für einen intermediären Adaptationsphänotypen des H5N1 HPAIV aus Accra. Desweiteren zeigten Pathogeneseuntersuchungen in der Maus, dass alle drei getesteten H5N1 HPAIV aus Ghana ohne vorherige Adaptation einen Gewichtsverlust sowie eine Letalität varrierend zwischen 40-100% verursachen können. Allerdings sind die in diesem Tierversuch verwendete Anzahl der Mäuse klein und eine Bestätigung mit einer größeren Tiergruppe ist erforderlich, um signifikante Aussagen über die Virulenz der einzelnen H5N1 Isolate aus Ghana treffen zu können. Auch sind weitere Untersuchungen notwendig, weitere adaptive Mutationen im Virusgenom zu identifizieren, die zu einer hohen Polymeraseaktivität sowie replikativen Fitness in humanen Zellen führen. Zusammenfassend kann man sagen, dass diese Untersuchungen erste Hinweise darüber liefern, dass die in Ghana zirkulierenden aviären IAV bereits human-adaptive Eigenschaften zeigen. Diese Befunde sprechen auch dafür, dass in Afrika ein Surveillance Programm gegen zoonotische Influenza an der Grenze zwischen Tier und Mensch dringend notwendig ist. Erst dadurch kann rechtzeitig das pandemische Potenzial dieser neuartigen HPAIV bestimmt werden, um gegebenenfalls Gegenmaßnahmen rechtzeitig einzuleiten.

## Summary

In Ghana, morbidity and mortality due to influenza was considered negligible until the 2009 H1N1 pandemic and the recent H5N1 highly pathogenic avian influenza A virus (HPAIV) outbreak in poultry. The country subsequently initiated an influenza surveillance programme for the general human population. However, a surveillance programme at the animal-human interface assessing the zoonotic potential of circulating avian influenza viruses is still missing. In this study, we aimed to identify and characterize circulating IAV strains in Ghana regarding their zoonotic potential. Therefore, we performed a sero-surveillance study among farmers and animals. We found that farmers were sero-positive against 2009 pandemic H1N1 and H3N2 IAV but not against H5N1 HPAIV. Poultry and pigs assessed at the respective farms were all sero-negative for the analyzed human strains suggesting that no transmission occured from human-to-animal. However, only a few months later, the H5N1 HPAIV poultry outbreak occurred in Ghana in 2015 resulting in significant economic losses. The outbreak was finally contained by stamping out over 100,000 poultry that put the country´s food security at risk. To analyze whether the H5N1 HPAIV strains might have acquired adaptive mutations in their genome possessing the potential to jump species barriers and infect humans, we have isolated these outbreak strains from several affected sites in Ghana. Phylogenetic analysis of the Ghanaian H5N1 HPAIV revealed that they harbor the polymerase subunit PB2 of H9N2 avian influenza viruses leading to their classification as the WHO clade 2.3.2.1c with known potential for human transmission. Interestingly, all isolated Ghanaian strains were closely related to H5N1 HPAIV 2015 outbreak strain from Nigeria with 80-99% amino acid homology. These findings suggested that the 2015 HPAIV strains in Ghana were likely introduced from Nigeria, where a similar outbreak was reported a few months earlier, probably by poultry movement between the countries. We then continued to analyze the Ghanaian H5N1 outbreak strains (isolated from Accra, Ketu and Obuasi) regarding their pandemic potential by assessing known molecular parameters of cross species transmission *in vitro* and *in vivo*. The viral receptor binding protein HA showed specificity to binding of avian-type α2,3-linked sialic acid containing receptors suggesting that they have not acquired the ability to bind to the human-type α2,6-linked sialic acid containing receptors yet. However, particularly the Accra H5N1 isolate presented high viral polymerase activity in human cells compared to an avian H5N1 outbreak strain isolated from birds in Germany (R65). In line, the H5N1 Accra isolate also displayed an increased replicative fitness in human cells, where they replicated to higher titers than the avian-type R65 but still lower than a human H5N1 isolate obtained from a fatal case in Thailand (KAN-1). Finally, all Ghanaian strains were pathogenic in mice without prior adaptation. The Obuasi strain showed highest pathogenicity with 100% lethality in mice

similar to KAN-1, while the Ketu and Accra strains showed 40-50% lethality. In summary,our findings suggest that the 2015 Ghanaian outbreak strains have already acquired human-adaptive signatures, which highlights their potential for zoonotic transmission from animal-to-man. Future studies are required to identify specific mutations in the viral genome that mediate elevated polymerase activity and replicative fitness in human cells. This information will be invaluable to establish and improve influenza surveillance systems at the animal-human interface not only in Ghana but worldwide.

## Abbreviations

| | |
|---|---|
| °C | degree Celsius |
| µg | microgram |
| µl | micro litres |
| µM | micro Molar |
| AIDS | acquired immune deficiency syndrome |
| ANOVA | analysis of variance |
| AUG | adenine uracil guanine |
| bp | base pair |
| cDNA | complimentary deoxyribonucleic acid |
| crm1 | chromosome region maintenance 1 |
| cRNA | copy ribonucleic acid |
| C-terminus | carboxyl terminus |
| DNA | deoxyribonucleic acid |
| $ddH_20$ | double distilled water |
| dNTPs | deoxy nucleotide triphosphates |
| e.g. | example |
| g | relative centrifugal force |
| h.p.i | hours post infection |
| $H_2O$ | water |
| HIV | human immunodeficiency virus |
| Hsp90 | heat shock protein 90 |
| Hz | Hertz |
| ie | that is |
| IFN | interferon |
| IgA | immunoglobulin A |
| IgG | immunoglobulin G |
| IL-4 | interleukin-4 |
| IL-6 | interleukin-6 |
| kg | kilograms |
| Log | logarithm |
| MCP-1 | monocyte chemoattractant protein-1 |
| mg | milligram |
| ml | millilitre |
| mM | milli Molar |
| mm | millimetre |

| | |
|---|---|
| MOI | multiplicity of infection |
| mRNA | messenger RNA |
| mU | micro Units |
| ng | nanogram |
| nm | nanometre |
| N-terminus | amino-terminus |
| p.f.u. | plaque forming units |
| P1 and 2 | passage 1 and 2 |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| pH | power of hydrogen |
| qPCR | quantitative polymerase chain reaction |
| RanBP5 | RAN-binding protein 5 |
| RANGTP | ras-related nuclear protein Guanosine Triphosphate |
| RANGTPase | ras-related nuclear protein Guanosine Triphosphatase |
| RIG-I | retinoic acid inducible gene I |
| RNA | ribonucleic acid |
| Th1 | type 1 T helper |
| TNF-α | tumour necrosis factor-1 |
| TPCK | tosyl phenylalanyl chloromethyl ketone |
| U/µl | units per micro litre |
| US | United States |
| UV | ultra violet |
| V | volts |
| vRNA | viral RNA |
| vRNPs | viral ribonucleoproteins |
| WHO | World Health Organization |

**amino acids**

| | |
|---|---|
| A | alanine |
| C | cysteine |
| D | aspartic acid |
| E | glutamic acid |
| F | phenylalanine |
| G | glycine |
| H | histidine |
| I | isoleucine |
| K | lysine |

| | |
|---|---|
| L | leucine |
| M | methionine |
| N | asparagine |
| Q | glutamine |
| R | arginine |
| S | serine |
| T | threonine |
| V | valine |
| Y | tyrosine |

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## 1.1 Introduction

Influenza is an acute febrile self-limiting viral infection of the upper respiratory tract and a disease of global public health significance. It affects people of all age groups, in both developing and industrialized countries. Symptoms include: sudden onset of fever, headache, cough, body aches, sore throat, sneezing, nasal congestion and general feeling of malaise. Influenza can cause severe illnesses and death among risk groups. It is estimated that there are about 3 to 5 million cases of severe illnesses caused by influenza A viruses (IAV) every year leading to about 300 000 to 500 000 deaths worldwide [1-5].

IAVs are enveloped viruses that belong to the family of *Orthomyxoviridae.* Members of this family are defined by segmented, single-stranded, negative sense RNA genome [6]. The family consists of 7 different genera: the influenza viruses A, B, C and D, Isaviruses, Thogotoviruses and newly proposed Quarjaviruses (made up of Quaranfil, Johnston Atoll and Lake Chad) viruses [6-8]. IAVs are responsible for seasonal infections [6] as well as occasional pandemics in the human population, leading to elevated mortality rates [9, 10] and therefore all further discussions will focus on IAVs. They are further divided into subtypes based on their surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA) into 19 (H1-19) HA subtypes and 11 (N1-11) NA subtypes [11, 12]. All IAVs (with the exception of H17-19 and N10-11) are known to circulate in wild aquatic birds [6]; they are therefore considered the natural reservoirs of IAVs. Virus strains are currently named according to the following nomenclature system [13]: their genus (influenza A), species from which it was isolated (eg. chicken), this is omitted when it is human, location where it was isolated, number of the isolate, year of isolation and the subtype in parenthesis. For eg. A/swan/Germany/R65/2006 (H5N1) signifies a H5N1 virus, isolated from swan in Germany in 2006 and was the R65$^{th}$ isolate.

### 1.1 Influenza A virion structure and organization

IAVs are known to be pleomorphic, with shapes ranging from roughly spherical to filamentous forms. Spherical forms have diametres ranging from 80-120nm and filamentous forms are up to 300nm in length. IAVs possess a lipid membrane derived from the host cell [6]. A schematic representation of the IAV virion is shown in Figure 1. Embedded in the envelope are the surface glycoproteins HA and NA as well as the matrix 2 (M2) proteins. The HA and NA surface glycoproteins appear as distinctive spikes on the surface of the virion with the ratio of HA to NA being approximately four to one [6]. The amount of M2 ion channels present on the lipid membrane compared to HA is minimal, with the ratio of M2 to HA being 1:10$^1$ to 10$^2$ HA molecules [6]. Directly beneath the viral envelope lies the matrix 1 (M1) protein which encloses the core of the virion. The core of the virion contains the

ribonucleoprotein (RNP) complexes which comprise the viral RNA segments, each associated with the polymerase segments polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acid (PA) and the nucleoprotein (NP). Present in the virion core is also the nuclear export protein/nonstructural protein 2 (NEP/NS2) [6] as well as the previously considered non-structural protein NS1 [14].

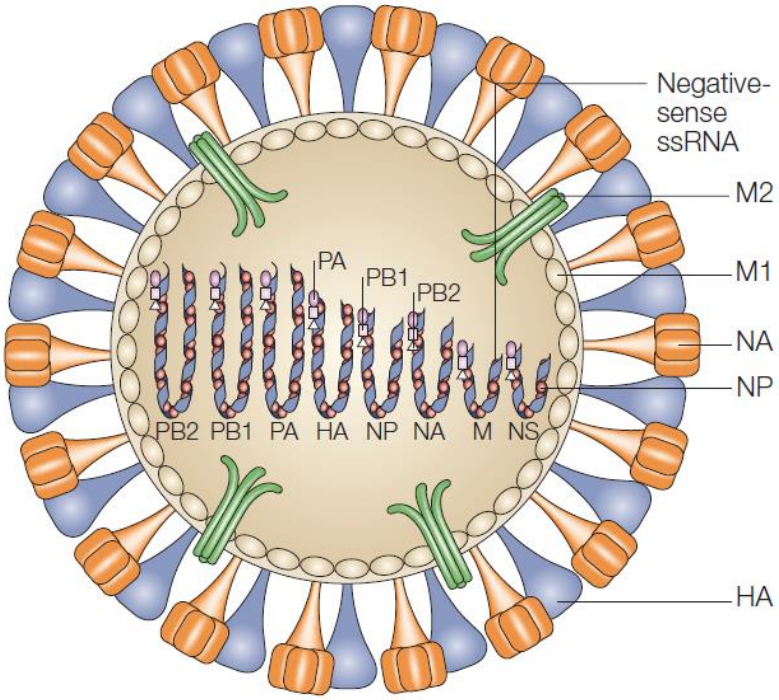


**Figure 1. Schematic diagram of an influenza A virion**. The virus derives its envelope from the host cell. Two surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) project from the surface of the virion. Also embedded in the envelope are the matrix 2 (M2) proteins. In the core of the virion are the ribonucleprotein complexes which comprise of RNA segments each linked to the polymerase proteins polymerase basic 1 and 2, (PB1 and PB2), polymerase acid (PA) as well as nucleoprotein (NP). Matrix 1 (M1) protein encloses the core of the virion which also contains the nuclear export protein/nonstructural protein 2 (NEP/NS2). (credit: Horimoto T, Kawaoka Y., Nature Review 2005)[9].

### 1.1.1 The viral surface glycoproteins

Embedded on the surface of the IAV are the surface glycoproteins HA and NA with a small amount of M2 ion channels which traverse the lipid envelope [6]. The HA is a trimeric, rod-shaped, type I integral membrane protein. Its carboxyl terminus is implanted into the viral membrane but the hydrophilic end projects as a spike on the viral surface. It is first synthesized in infected cells as a single polypeptide chain (HA0) which is then cleaved into HA1 and HA2 which appear as different subunits. This cleavage is essential for the fusion activities of HA [15, 16]. The HA1 forms the globular head of the glycoprotein and is mainly responsible for the binding of HA to cell surface receptors to initiate cellular entry, with the

receptor binding site located in the globular head [17]. The receptor binding site is described by three domains: the 130-loop (amino acid residues 134-138), the 190-helix (amino acids residues 188-195) and the 220-loop (amino acid residues 221-228) [17, 18]. Also found in the globular head of HA are the major antigenic epitopes to which host antibody responses are directed [19]. The HA2 forms a long fibrous stem and contains a stretch of conserved 20 amino acids in its N-terminus. This sequence is referred to as the fusion peptide and is responsible mainly for triggering fusion between the viral envelope and the host cell membrane, providing vRNPs access to the cytoplasm of an infected cell [6, 17]. The HA also contains a cytoplasmic tail whose function is not yet well defined due to subtype and host type specific differences [20, 21]. A schematic representation of the IAV HA protein is shown in Figure 2. The second major glycoprotein on the surface of the IAV is the NA. The NA is a type II integral membrane protein with a hydrophobic transmembrane region as well as a highly conserved short cytoplasmic tail [22]. The NA functions to cleave sialic acids from the surface of infected cells, thereby releasing newly formed virus particles [22, 23]. Oseltamivir and Zanamivir which are sialic acid analogues target IAV NA to slow down the release of virus from infected cells [24]. M2 proteins, which are tetrameric type III integral membrane proteins and lack a signal peptide, are also found on the surface of the virion. They function to moderate the pH within the virion and weaken the bond between the vRNPs and the M1 protein, leading to release of vRNPs into the cytoplasm [24].

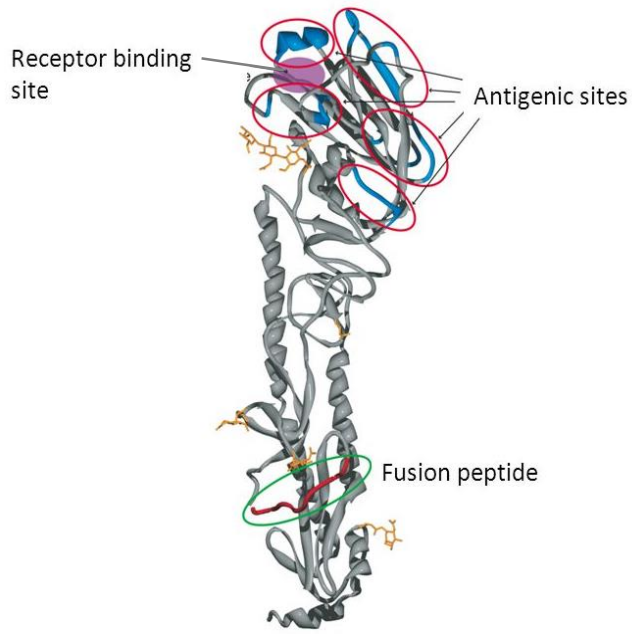


**Figure 2. Schematic representation of influenza A virus HA monomer**. The monomer is shown in grey with antigenic sites shown in red circles. Fusion peptide is shown in red with receptor binding site shown as pink circle. Credit: Stevens *et al*., 2003 [25].

### 1.1.2 The viral core

The IAV core contains eight RNA segments which code for at least one protein per RNA segment [6]. The RNA segments do not exist as naked RNA, rather each segment is complexed with four viral proteins to form a vRNP (viral ribonucleoprotein) complex. Proteins that make up the vRNP complex include the nucleocapsid protein NP and the three polymerase proteins PB2, PB1 and PA. An overview of the genomic structure of influenza A/Puerto Rico/8/1934 (H1N1) virus showing details of gene segments and the proteins each gene segment codes for is shown in Figure 3. Although the genome size is limited, IAV is able to achieve effective replication in susceptible host cells by employing several different viral proteins. The virus is able to utilize different mechanisms to encode one or more viral proteins from single gene segments. Examples include M2 and NEP/NS2 proteins which are produced by splicing of M and NS genes, respectively [6]. Several novel proteins have since been identified due to such occurrences. The novel PB2-S1 proteins were found to be encoded by a spliced mRNA from the PB2 segment. It was shown to localize to mitochondria, inhibited the RIG-I-dependent interferon signaling pathway and interfered with viral polymerase activity [26]. IAV PA-X protein, another newly discovered protein is encoded by a second open reading frame in segment 3 of the virus (X-ORF). The PA-X protein was found to repress cellular gene expression and modulated host responses to influenza infection [27]. Newly identified PA related proteins: PA-N155 and PA-N182 were found to be encoded by the $11^{th}$ and $13^{th}$ AUG codons in segment 3. While the proteins did not exhibit any polymerase activity together with PB1 and PB2, they probably have important functions in the IAV replication cycle [28]. The NS3 proteins, discovered recently, were identified during adaptation of an avian virus to a mouse host. During the process, the NS1 A374G substitution, led to a D125G coding mutation which triggered a new splice site, leading to the translation of NS3 proteins. The NS3 protein sequence, though similar to NS1 protein, contains an internal deletion starting from amino acid positions 126 through to 168. It was observed that NS3 proteins led to increased viral replication in mice [29].

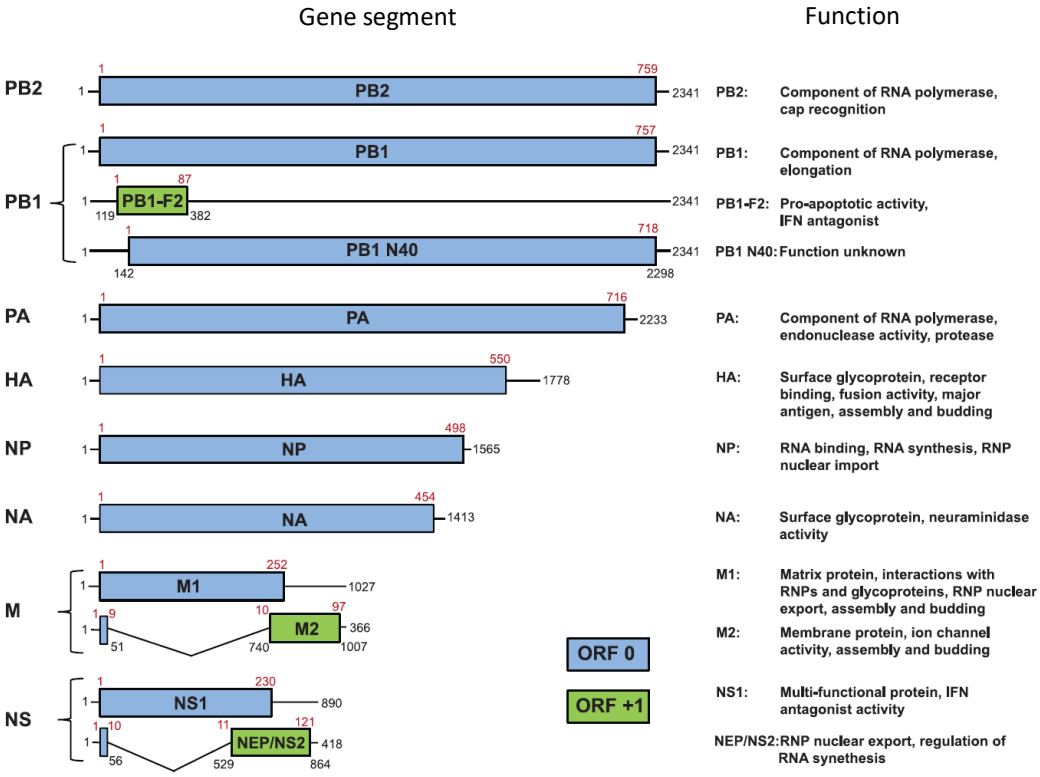


**Figure 3. Outline of the genomic structure of influenza A/Puerto Rico/8/1934 (H1N1) virus, showing functions of various encoded proteins**. Amino acids of RNA segments (RNA segments are shown in black) are indicated in red. Non coding regions are indicated with black lines at the 5´ and 3´ ends. Introns are represented with v-shaped lines (credit: Lamb R. A, Krug R. M., Fields Virology, 6th edition)[6].

## 1.2 Influenza A virus replication

### 1.2.1 Receptor binding and entry into host cells

IAVs infect epithelial cells in the upper and lower respiratory tracts of humans [6]. A schematic representation of the IAV life cycle is shown in Figure 4 [30]. To initiate infection, IAV HA binds to sialic acids on the surface of susceptible cells preferentially through SAα2,3Gal for avian influenza viruses, or SAα2,6Gal for human adapted viruses [17, 31-33]. The virus is then internalized into the cell via clathrin-mediated endocytosis [34, 35]. This is the method widely reported for influenza virus entry into host cells although other ways have also been reported [6, 36]. The internalized virion escapes degradation by hydrolytic enzymes and travels through the cytoplasm enclosed in an endosome [34, 35]. As the internalized virus travels through the cytoplasm, the pH inside the endosome reduces (pH 5-6). This low pH is maintained by proton pumps within the endoplasm. At a pH threshold in the late endosome, a conformational change in the HA spike is triggered. This change is irreversible and exposes the 'fusion peptide' at the N- Terminus of HA2. This enables it to interact with the endosomal membrane, inducing a merger of the endosomal membrane with the viral membrane. The presence of several HA molecules present at a time, leads to the

creation of a pore through the endosome [37]. The pH at which the conformational change is triggered differs between avian viruses and human adapted viruses. For avian viruses, this happens at pH of approximately 5.5-6.0, whereas for human adapted viruses such as H1N1 and H3N2, this occurs at pH approximately 5.0-5.5 [38-40]. The release of vRNPs from the virion core is mediated by M2 proton channel. Protons from the low acidic environment of the endosome flow into the viral core, disrupting the bonds between the vRNPs and the M1 layer, leading to a release of vRNPs into the cytoplasm [41]. The antiviral drugs Amantadine and Rimantadine have been shown to block the ion channel activity of M2 [42]. The uncoating process is completed when free vRNPs appear in the cytoplasm of an infected cell [43].

### 1.2.2 Transport of vRNPs into the nucleus of infected cells

IAVs transcribe and replicate their genome in the nucleus of infected cells [6]. Each RNA segment is coated with NP and forms a helical hairpin which is bound on one end by the polymerase complex units: PB2, PB1 and PA. This conformation is usually referred to as a cockscrew structure and is too large to simply diffuse into the nucleus passively and needs to be actively transported into the nucleus. Transport of proteins into the nucleus is an energy dependent process and utilizes members of the importin-α (karyopherin-α) family [44] The virus uses the classical importin-α/$\beta_1$ pathway to transport vRNPs into the nucleus. All proteins in the vRNP complex possess nuclear localization signals (NLS). It has been shown that, the NLS signal from NP is sufficient for the import of vRNA [45, 46]. PB2 and NP are also most likely imported this way through direct interaction with importin-α isoforms. Once there is interaction between the NLS of the cargo protein and the adaptor protein, the importin-$\beta_1$ transporter receptor is recruited. This ternary complex: importin-$\beta_1$-importin-α-cargo then passes through the nuclear pore complex. Inside the nucleus, importin-$\beta_1$ is dissociated by an activated form of RAN GTPase (RAN-GTP), leading to the release of importin-α-cargo. Importin-α is released from the cargo through the use of a cellular protein after, which both importin-$\beta_1$ and importin-α are individually recycled back into the cytoplasm. PB1 and PA are transported through a non-classical route by binding directly to the importin-β homologue RanBP5. It has been suggested that PB1-PB2 or PB1-PA are transported into the nucleus by Hsp90 [47-49]. To date, six importin-α isoforms are known with homologies between chicken and humans [50-52]. The amino acid similarities between human and chicken importin-α isoforms vary from 82-99% [53]. Gabriel and colleagues (2011) demonstrated that IAVs differentially utilize importin-α isoforms; while avian viruses preferentially bind to importin-α3, human viruses preferentially utilized importin-α7, with both viruses using importin-α1 [53].

### 1.2.3 Replication and translation and of the viral genome

Inside the nucleus of the infected cell, the negative-sense RNA genome of IAVs is first transcribed into mRNA. This requires the use of a primer, which the virus steals from the cap of host pre-mRNA transcripts from cellular RNA polymerase II. This mechanism is referred to as 'cap snatching' [63]. Synthesis of cellular proteins is therefore inhibited in favour of viral proteins. Transcription is initiated when the 5'-end of vRNA binds to the PB1 subunit. This enables PB2 to recognize and bind to the cap structure on host pre mRNAs and through endonuclease activation, cleave the cap from pre-mRNA [54, 55]. Once the cap is cleaved, addition of a 'G' residue to the primer initiates transcription [55]. Polyadenylation of viral mRNA transcripts is catalysed by the viral polymerase [56]. The mRNAs are transported into the cytosol for translation by cellular ribosomes. Newly synthesized proteins associated with the vRNP complex are transported back into the nucleus for assembly of progeny vRNP complexes after their translation is complete [57]. The negative sense vRNA serves as templates for both mRNA transcription and replication of full-length complementary genomic positive sense-RNA (cRNA). cRNA directs the synthesis of several new copies of negative-sense vRNAs. Synthesis of mRNA and cRNA are both catalysed by the viral RNA-dependent RNA polymerase but the switch from transcription to replication is poorly understood. It has however been proposed that the availability of soluble free NP may be responsible for controlling the switch [58] and NP has also been implicated in the synthesis of full length cRNA [59]. mRNA transcripts and proteins are transcribed and translated disproportionately, largely dependent on their roles at different points in the viral life cycle. NP and NS1 proteins are translated early; NP is required for RNA replication while NS1 is known to play a role in combating the host immune responses. NP and NS1 proteins are therefore translated early. HA, NA and M1 are translated late because they are needed much later in the viral life cycle. M1 is known to be involved in export of vRNP from the nucleus and is therefore required after vRNPs are present [58, 60]. Nuclear export/non-structural 2 proteins are involved in the export of newly formed vRNP complexes out of the nucleus into the cytoplasm [61]. NEP/NS2 proteins associate with Crm1 (export receptor) [62] and several nucleoporins [63] as well as with M1. It has therefore been proposed that a vRNP:M1:NEP/NS2 complex is formed, with the NEP/NS2 protein binding to Crm1, leading to an export of vRNPs outside of the nucleus [63]. This complex is then dissociated in the cytoplasm.

### 1.2.4 Assembly and release of progeny virus

Newly formed viruses assemble and bud from the plasma membrane of an infected cell. Viral envelope proteins HA, NA and M2 are translated in the cytoplasm and transported to the endoplasmic reticulum where the proteins (apart from M2) are folded and glycosylated. HA is subsequently assembled into a trimer and NA and M2 into tetramers. The proteins are then transported through the Golgi apparatus and trans-Golgi network to the plasma membrane [6, 64]. The dissociated M1 protein then interacts with HA and NA that are now fully embedded in the plasma membrane, forming patches. Newly formed vRNPs then actively interact with these M1 patches, preventing re-entry of vRNPs into the nucleus. Correct packaging of vRNPs is essential to ensure that a fully infectious virion is released. Exactly how viral ribonucleoproteins are packaged is not fully understood but two models have been proposed. The first model describes a mechanism where vRNPS are packaged randomly; here there is a common motif on all vRNPs that allow them to be randomly packaged [65]. This model is referred to as the random incorporation model and has been difficult to prove. A second model, the selective incorporation model, suggests that each vRNP has a unique packaging signal so that packaging will favour the formation of an infectious virion and there is growing evidence to support this theory [66-70]. M1 proteins drive formation of a bud of newly assembled virions at the plasma membrane of an infected cell [71]. Budding is complete when the viral envelope has separated from the cell surface. However, after budding, newly formed virions are still anchored to the cell surface through interaction of HA with sialic acid containing cell surface receptors and need to be actively released. Viral NA cleaves the sialic acids on the cell surface, leading to a release of newly formed virions [24].

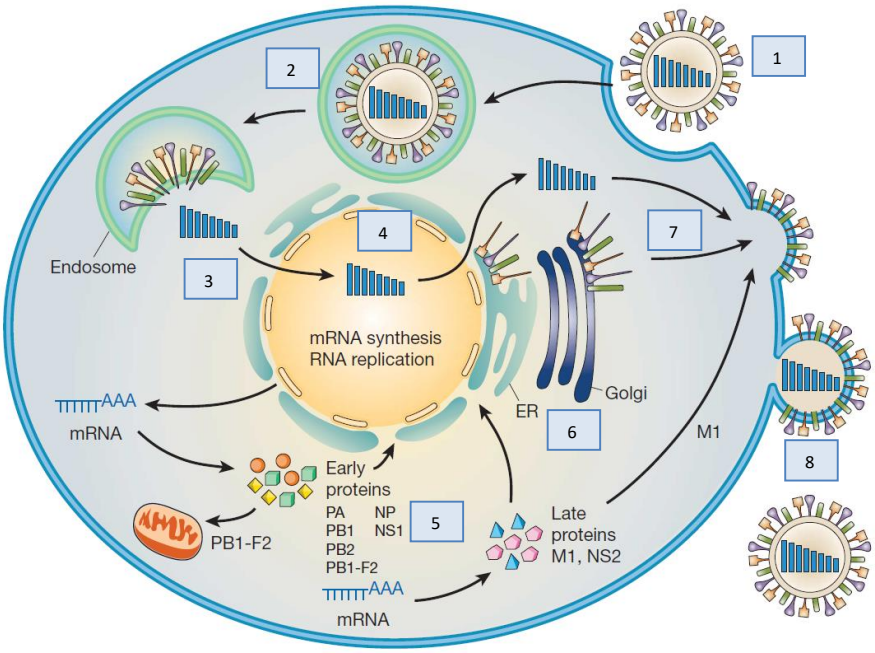


**Figure 4. Schematic representation of the influenza A virus life cycle**. IAVs enter host cells through interaction of HA surface glycoproteins with either SAα2,3Gal or SAα2,6Gal present on host cells (1). Viruses are then internalized and travel through the cytoplasm in an endosome (2). In the late endosome, a comformational change in HA leads to a fusion of the viral envelope with the endosome. Acidification of the virion core, leads to a weakness in the interaction of vRNP with the M1 protein and release of vRNPs into the cytoplasm which are then actively transported into the nucleus (3). Negative sense vRNA acts as a template for both primary transcription and replication (4). mRNA transcripts are exported to the cytoplasm for translation. Early transcripts PB2, PB1, PA, NP needed for the assembly of new vRNP complexes are transported back into the nucleus (5). Other early transcripts include NS1 and PB1-F2 proteins. HA, NA and M2 after translation are modified in the ER and transported through the golgi network to the plasma membrane (6). Late proteins NS2/NEP form a complex with M1 to transport newly formed vRNPS into the cytoplasm. Exported vRNPS interact with M1 and are packaged into progeny virus particles. (7). NA cleaves HA on the cell surface to prevent aggregation of newly formed virus particles and thereby releasing them to infect other cells (8). (credit: modified from Neuman G, Noda T, Kawaoka Y., Nature Review, 2009) [30].

### 1.3 Epidemiology of influenza A viruses

IAVs are known to infect a wide range of animals; including humans, pigs, birds, cats, dogs, seals and horses (Figure 5) [6, 9, 72]. With the exception of H17-19 and N10-11, all influenza subtypes have been isolated from wild aquatic birds and therefore they are considered as the natural reservoirs of IAVs [6]. These wild aquatic birds are of the order *Anseriformes* (ducks, geese, swan) and of the order *Charadriiformes* (gulls, terns, surfbirds, sandpipers) [6]. In ducks, IAVs replicate mainly in the epithelial cells in the intestinal tract leading to the virus being shed in large amounts in feces [73]. IAVs have been isolated from water bodies where wild birds have been known to have congregated before migrations [74]. Although, IAVs continue to acquire mutations in the natural aquatic host reservoir, these mutations do not result in a selective advantage for the virus. The viruses cause mild asymptomatic infections in aquatic ducks. Due to this asymptomatic nature of the infection in aquatic ducks (natural

hosts) and the fact that the virus seems to be in an evolutionary stasis in this avian host, it has been suggested that the virus is optimally adapted to these hosts [6]. Avian IAVs can be classified as low pathogenic or highly pathogenic based on their pathogenicity in chickens. Low pathogenic avian influenza viruses (LPAIV), cause only mild respiratory disease in chicken and may lead to depression/low egg production. In the avian reservoir, LPAIV replicate largely in intestinal and respiratory organs with viruses being shed in the faeces of infected birds. Therefore, LPAIV are transmitted mainly through the fecal-contaminated-water-oral route among aquatic birds [9]. An IAV is classified as highly pathogenic (HPAIV) when it is lethal for 6-8 out of 8, 4-8 week old susceptible chickens infected with 0.2ml of $10^1$ infective allantois fluid among other characteristics [6]. Most HPAIVs possess a series of basic amino acids at their HA cleavage site. Known HPAIV to date all belong to H5 and H7 subtypes [6]. For the natural reservoir, even though high concentrations of HPAIVs are shed in feces of infected birds, viruses are more easily transmitted by the nasal and oral routes among birds in densely populated flocks [9]. When avian IAVs are introduced into land based poultry or mammalian species, because of a lack of immunity of the new host, different disease symptoms and manifestations of the disease may occur. There is a different selection pressure exerted on the virus leading to mutations in the viral genome. These mutations, if beneficial to the fitness of the virus, can lead to adaptation of the virus to the new host [10].

IAVs replicate in epithelial cells lining the upper respiratory tract of the human airways. IAVs H1, H2 and H3 subtypes, which are normally isolated from humans are thought to have been originally transmitted to the human host and are now fully adapted to cause infections in humans. [6]. Viruses are maintained in the human population through direct human to human transmission. The most effective mode of transmission is through aerosols. Aerosols produced during sneezing or coughing are <2µm in diametre and are easily deposited directly in the respiratory airway [75] for onward transmission.

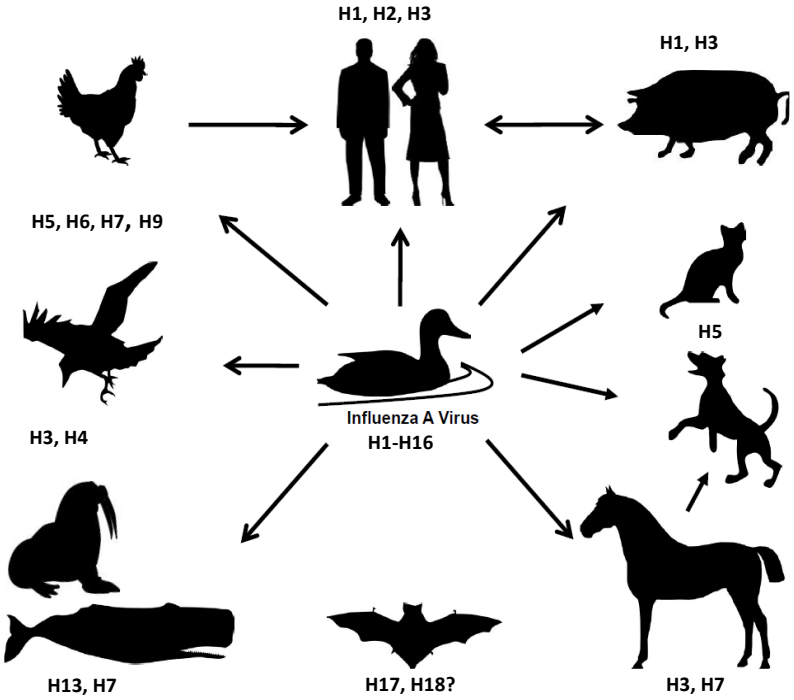


**Figure 5. Host range diversity of influenza A viruses.** Wild aquatic ducks are thought to be the natural hosts of influenza A viruses. From here, they can be transmitted to a wide range of animals including: humans, pigs, dogs, cats, domestic poultry, seals, horses, pigs. There have also been reports of novel HA subtypes isolated from bats (credit: modified from Mänz B., Schwemmle M., Bunotte L., Jour of Virol, 2013) [72].

### 1.3.1 Influenza epidemics and pandemics

IAVs undergo constant antigenic evolution. Because of this, they are able to evade host immunity so that previous responses elicited against the virus fail upon infection with a newer variant. The HA is the major antigenic component to which antibodies are directed. Consequently, it is the HA protein that undergoes antigenic evolution and mainly responsible for the evasion of the host immune system [6]. Other viral proteins also undergo less variation and may contribute to immune evasion [76]. Antigenic evolution primes the occurrence of annual influenza epidemics, which necessitates the need for constant updates of the composition of the influenza vaccine. There are two main mechanisms by which antigenic evolution occurs namely antigenic drifts and antigenic shifts [6].

### 1.3.2 Antigenic drifts - epidemics

Antigenic drifts arise as a result of point mutations, minor and gradual in the HA protein [6]. The RNA-dependent RNA polymerase of the virus is error-prone and lacks proof reading mechanisms. These lead to a mutation frequency of 1 in 100 000 nucleotides. Although most of these mutations could be silent or lead to stop codons, some could lead to variant viruses

with selective advantage over the parent virus. Antigenic drift in HA is therefore a result of accumulation of point mutations in the antigenic epitopes of HA. These mutations could lead to newer variants, which are no longer neutralized by existing antibodies [77]. Antigenic drifts lead to annual influenza epidemics where estimated 50 million illnesses are reported every year in the US alone. Influenza epidemics account for the annual recorded deaths of 300 000 to 500 000 people worldwide [6]. In temperate countries, influenza epidemics typically occur during winter months: October to April, while in tropical and sub-tropical countries, it coincides with the rainy seasons [6].

### 1.3.3 Antigenic shift - pandemics

Antigenic shift describes the phenomenon where new IAV proteins are introduced to the human population. These proteins are antigenically novel from circulating strains, with the general population being immunologically naïve to these proteins, triggering pandemics. These outbreaks impact large geographical areas and occur on more than one continent at the same time, leading to very high infection rates among very large numbers of people with high mortalities. In the past century, the world has recorded 5 pandemics: in 1918 (‘Spanish influenza’ caused by the introduction of H1N1), in 1957 (‘Asian influenza’ - H2N2), 1958 (‘Hong Kong influenza’ - H3N2), 1977 (‘Russian influenza’ - H1N1) and in 2009 (‘Swine influenza’ - H1N1). There are three ways by which new viral proteins can be introduced into the human population. They are: a) reassortment events. Here, an avian virus could reassort with a human strain and/or swine strains, leading to new variants. The pandemics of 1957 and 1968 were caused by human and avian reassortant viruses. The virus that caused the 2009 pandemic was a triple reassortant virus; with gene segments from swine, human as well as avian strains. b) Through the direct transmission of an avian virus into the human population. Such a virus then quickly establishes in the population and begins to cause infections. Phylogenetic analysis suggests that, the 1918 Spanish influenza pandemic which claimed the lives of ~50 million people was an avian virus which was directly transmitted to humans. c) Reintroduction of a human strain that was previously in circulation. An example is what is now believed to be an accidental re-introduction of H1N1 in 1977 causing the ‘Russian influenza’ [6, 78].

## 1.4 Highly pathogenic avian influenza viruses in Africa

In 2012, Dawood and colleagues estimated that 201 200 respiratory-related as well as 83 000 cardiovascular-related deaths occurred due to the 2009 influenza pandemic. Out of these, 51% would have occurred in Africa and south East Asia [79]. The impact of influenza disease burden, epidemiology and seasonality in Africa has been considered negligible because the continent is plaqued by poverty, poor living standards and high burden of

diseases such as HIV and AIDS, tuberculosis and malaria [80, 81]. Governments and relief efforts have subsequently focused on these.

History of influenza in sub-Saharan Africa however dates as far back as 1891 when the pandemic of 1889-1893 reached modern day Ghana (then Gold Coast) in 1891, causing a number of deaths with recorded cases in the subsequent years [82]. Again from 1918 to 1919, large numbers of deaths (probably more than 100 000 in a population of 4 million) were recorded in the then Gold Coast when the 1918 pandemic was introduced to the country. Reports of outbreaks were consequently reported in almost all countries in sub-Saharan Africa, leading to an estimated 1.5-2 million deaths in the region. The infection was thought to have most likely been introduced by ships coming from England and landing in Sierra Leone, subsequently moving on to the then Gold Coast (modern day Ghana) [82, 83]. These records were kept by colonial physicians and after countries gained independence and started managing their own affairs, proper influenza surveillance systems were not set up or maintained.

In 2011, a systematic review of published data on influenza from 1980-2009 in sub-Saharan Africa was conducted to understand influenza disease burden and epidemiology in this region. It was observed that out of the 46 WHO African countries, only three: South Africa, Senegal and Madagascar routinely reported influenza activity to WHO, with Zambia, Uganda, Mauritius, Reunion and Kenya reporting sporadically [84]. This observation has dire consequences because in a region where the nutritional status of the population is poor, access to good health care is pitiable, heavy disease burden and vaccines as well as antibiotics are sometimes not easily accessible, an influenza pandemic could be devastating. Coupled with the above mentioned factors, death due to respiratory factors among young children in Africa are reported to be the highest in the world [81, 85]. Africa has a fairly young population (average age 19.4 years) with young people <15 years making up 41% of the population [86]. In case of an influenza pandemic, Africa will be the hardest hit. The narrative of influenza surveillance in Africa has however in the past 10 years, made a substantial turn around. Two factors have accounted for these: first, outbreaks of HPAIV of the subtype H5N1 among poultry in some African countries causing enormous poultry deaths leading to huge economic losses for affected countries as well as human deaths [81]. HPAIV of the subtype H5N1 was first recorded among chicken in sub-Saharan Africa in February 2006 in Kaduna State in Northern Nigeria when viruses of the clade 2.2 were introduced to sub-Saharan Africa [87]. The virus spread very rapidly to countries like Egypt, Cameroon and Niger. A few months later in April 2006, there were reports from Burkina Faso, Djibouti, Sudan and Ivory Coast. The virus circulated widely in sub-Saharan Africa for about a year

with further outbreaks among chicken reported in Ghana, Togo (May, June 2007) and Benin (December 2007) [88]. This virus also caused human deaths in Africa. Deaths were reported in Nigeria, Djibouti (1 death each) and Egypt. To date Egypt has the highest number of deaths related to H5N1 in the world [89]. Seven years later, HPAIV of the subtype H5N1 again reached sub-Saharan Africa, with Nigeria again reporting the first cases among poultry in January 2015 [90, 91]. These new viruses belonged to the clade 2.3.2.1c and spread rapidly to several West African countries including Niger, Burkina Faso, Ghana and Cote d'Ivoire [91, 92]. No human cases have been reported with this virus even though the virus is still circulating in the region. In addition to these H5N1 viruses, there are currently H5N8 viruses circulating among chicken in Nigeria and African penguins in Cape Town, South Africa, H9N2 among chicken in Ghana [93] and Burkina Faso [94]. The second factor which has led to a different narrative of influenza in Africa has been the outbreak of the 2009 pandemic influenza H1N1 among the population. At the time, there was fear that this was a devastating new virus [81]. These situations have led to various countries establishing influenza surveillance systems. A number of organizations including the WHO, the US Centers for Disease Control and Prevention (US-CDC), the US military, the US Department of Health and Human Services, the International Network of the Institute Pasteur (RIIP) and the National Institute for Communicable Diseases (NICD) in South Africa have partnered, supported and worked together with ministries of health from various African countries to provide support for pandemic preparedness and the establishment of influenza virus surveillance systems [81]. These efforts have led to a drive for most African countries to provide evidence for policy change towards influenza in their countries. All these efforts have yielded results. Where as in 2006 <10 countries were conducting routine influenza virus surveillance in their countries, the number has risen to 30, with 20 regularly sharing their data globally [95]. The Journal of Infectious Diseases in 2012, created a supplement to focus mainly on influenza surveillance efforts in Africa. It included articles from 16 African countries which highlighted the impact of the pandemic of 2009 in the various countries in addition to reports from countries that were undertaking surveillance activities at the time. The articles discussed how the pandemic of 2009 affected different countries, response efforts by the countries and how the pandemic of 2009 made a delayed entrance into Africa. Other articles described knowledge about the pandemic influenza vaccine among the population and how challenges were overcome in receiving and administering vaccines to high risk populations such as health care workers and pregnant women [81]. The US-CDC in collaboration with the NICD, South Africa in 2012 organized the first meeting of the African Network for Influenza Surveillance and Epidemiology (ANISE). ANISE provided a platform where African countries could share strategies and encourage other countries in the development of surveillance systems for IAVs. Currently, very few countries in Africa vaccinate against

influenza. This is because so far not enough evidence has been gathered to make a strong case for the introduction of a vaccine. Important epidemiological evidence such as seasonality is a challenge because influenza seasonality is not very pronounced in the tropics, with infections occurring year round. Consequently the time period within which to administer a vaccine is a challenge. But as many more countries are establishing surveillance systems country wide, this observation will soon change. A final challenge is financial. Most countries are reluctant to engage in these discussions because they will incur huge extra financial burdens. The solution to these problems still depends on the provision of strong evidence that influenza disease is a burden to Africa. Although it will take some time, Africa's efforts in combating influenza disease has made huge progress in the past 10 years, with many more countries interested in establishing surveillance systems. Increased developmental advancement across Africa such as the thriving poultry and pig industries, placing humans in greater proximity with these animals threaten the progress the continent has made in combating influenza disease. Recent studies to determine the prevalence of infection among pigs have yielded positive results [96, 97]. Adeola and colleagues in 2016, could detect that 4% of pigs in two locations in Ibadan Nigeria and also 4% of pigs in Kumasi, Ghana possesed antigens of a human H3N2 virus [97]. Therefore the fear of reassortment of currently circulating HPAIVs in the region to produce a pandemic virus is tangible [98] and therefore progress is expected to progress quicker.

### 1.5 Key viral factors involved in avian HPAIV transmission to man

Wild aquatic birds of the order *Anseriformes* (ducks, geese, swan) and of the order *Charadriiformes* (gulls, terns, surfbirds, sandpipers) are known to be the natural reservoirs of IAVs as discussed in 1.3. [6]. Upon transmission to the mammalian host, selection pressure is exerted on the virus that could lead to mutations in the viral genome. These mutations are sometimes very beneficial to the fitness of the virus and lead to adaptation of the virus to the new host [10]. Adaptations could have serious consequences as demonstrated by pandemics that have occurred in the last century. Apart from the 1977 Russian flu pandemic, all the other major pandemics were caused by viruses that acquired HA from an avian virus. This observation emphasizes the fact that clues that lead to the molecular changes associated with the emergence of a pandemic virus lie with the avian reservoir [10]. It is therefore imperative that the contributing factors leading to adaptation of the avian virus to the mammalian host be investigated. This could help prevent such catastrophic pandemics or mitigate their effects. The adaptation of an avian virus to a mammalian host is a complex process involving multiple genetic factors. However, few very important determinants have been identified:

### 1.5.1 The viral haemagglutinin

The first barrier that an avian IAV encounters upon infection of a mammalian cell is the plasma membrane. The HA binds to N-acetylneuraminic acid (sialic acids) present on host cell surface to initiate infection [17]. Human adapted IAV prefer binding to N-acetylneuraminic acid attached to the penultimate galactose sugar by an α2,6 linkage (SAα2,6Gal), while avian viruses prefer the α2,3 linkage (SAα2,3Gal) [17, 31-33]. In support of these findings, earlier studies showed that, the epithelial cells present in human trachea possessed mainly SAα2,6Gal [99]. However, recent studies of human epithelial cells from trachea/bronchial tissues, have demonstrated that, non-ciliated epithelial cells (this forms the majority of epithelial cells present in trachea/bronchial tissues) possess SAα2,6Gal whereas ciliated cells (the minority population present in trachea/bronchial tissues) possess SAα2,3Gal [99]. On the other hand, epithelial cells found in the trachea and intestines of duck contain SAα2,3Gal in abundance [100]. These observations provide explanations for the host restrictive nature of IAVs and the accidental infection of human hosts by avian viruses [100]. The distribution of SAα2,3Gal or SAα2,6Gal which are recognized by IAVs HA proteins are therefore a major determinant of the host range of the virus. SAα2,3Gal are expressed in diminutive quantities in the lower respiratory tract of humans, but not in the upper respiratory tract. An upper respiratory tract infection in humans however, will ensure efficient aerosol transmission among humans. This therefore poses a host restriction for an avian virus [17, 31-33, 99]. Although the HA protein of viruses from the 1918, 1957 and 1968 pandemics were of avian origin, they were able to recognize SAα2,6Gal and cause infections. These observations suggested that these viruses at a point in their adaptation process, switched receptor specificity from SAα2,3Gal to SAα2,6Gal [101, 102]. Therefore for an avian virus to effectively infect a mammalian host, replicate and efficiently transmit the infection, changes in the viral genome should lead to a switch in receptor recognition. This could be crucial for the generation of a virus with pandemic potential. Recent HPAIVs of the subtype H5N1 seem to have acquired some mutations in nature which have led to increased binding of HA to SAα2,6Gal (Table 1). Herfst and colleagues could show experimentally that H5N1 HPAIV serially passaged in ferrets could acquire mutations that rendered the virus airborne transmissible among ferrets [103]. Imai and colleagues also showed that viruses possessing the HA of H5N1 HPAIV with mutations in the globular head of the HA could also be airborne transmitted by aerosols to ferrets [104]. These two studies answered the question of whether a H5N1 HPAIV virus, could evolve such that the virus could gain the ability to become airborne transmissible between mammals without an intermediate host. Although the mutations described by these studies have yet to be identified in natural isolates, they have provided valuable information about the ability of H5N1 HPAIV viruses to cause pandemics. The HA glycoprotein also provides the virion with access to the cytoplasm of the host cell by

the induction of fusion of the viral membrane with the endosomal membrane. Fusion is made possible when the precursor HA molecule, HA0 is cleaved into HA1 and HA2 by host cell proteases. HA cleavage is therefore an important determinant of viral pathogenicity [17]. For LPAIVs, cleavage occurs at a single arginine residue [9]. Proteases often referred to as 'trypsin-like', are then capable of cleaving HA proteins with a mono-basic cleavage site. These trypsin-like proteases are however restricted to the respiratory tract and gastrointestinal tract (water fowl, terrestrial birds) and hence, viral replication is restricted to these areas [105, 106]. The cleavage sites of HPAIVs on the other hand contain a series of arginine residues (multiple basic amino acids) and this motif is easily recognized by abundantly expressed proteases such as furin and PC6 (pro protein convertase 6), which are available in a wide range of different host cells. HPAIVs are therefore able to cause severe, systemic disease in poultry [107, 108]. This implies that HA which can be easily and readily cleaved is important for high pathogenicity. H5N1 viruses that have caused death in humans usually possess multiple basic amino acids in their HA cleavage site. Based on these, it is evident that current HPAIVs of the H5 or H7 subtypes possess the ability to cause serious future pandemics. Table 1 shows selected summary of amino acid changes/motifs which have been identified in avian IAVs that have caused an effect on some biological properties of the virus [109, 110].

### 1.5.2 The viral polymerase complex

The IAV polymerase complex consists of PB2, PB1, PA and NP proteins and is responsible for transcription and replication of the viral genome inside the nucleus of an infected cell [6]. It has been shown that polymerases deriving from an avian IAV have reduced activity in mammalian cells. Several amino acid substitutions, which have led to a rescue of the polymerase activity of avian viruses in the mammalian host have been described. The most extensively described mutation in the polymerase complex is E627K in PB2, where at position 672 glutamic acid (E) is replaced by lysine (K). This mutation has been described in fatal human cases involving H5N1 infections [111, 112]. PB2 E627K was also described to enhance viral polymerase activity in a mouse adapted H7N7 virus [111]. A second PB2 mutation which has been described is D701N. This mutation has been shown to increase polymerase activity in H3N2 viruses [113], H7N7 [114] and H5N1 [115]. It was observed that the mutation acts in part by enhanced import of PB2 into the nucleus of mammalian cells [114]. Both E627K and D701N mutations are usually not detected in combination in isolates. This observation suggests that both signatures play a similar role and that their simultaneous occurrence in the protein would hinder their function [110, 114]. In addition to the poor activity of the polymerase of avian viruses in mammalian cells, the nuclear membrane also forms a major barrier that the virus needs to overcome [53, 56, 111]. A number of amino acid

substitutions present in the viral polymerase complex which have led to increased replication as well as a differential use in the host factor importin-α isoforms have been described. As discussed in section 1.3.2, several importin-α isoforms present in the host cell, act as adapter proteins, mediating nuclear import of vRNPs [50-52, 54]. It has already been shown that both avian and human adapted viruses require the use of importin-α1 while avian viruses preferentially utilize importin-α3 and human adapted viruses importin-α7 [53]. This observation once more highlights the importance of the importins in the avian mammalian adaptation process. Another important mutation that has been described in the viral polymerase is in NP N319K which usually occurs together with D701N in PB2. This mutation has been shown to enhance polymerase activity in mammalian cells as well as increase pathogenicity in mice, by enhancing binding to importin-α isoforms [110, 114]. Several other compensatory mutations in the RNA-dependent RNA polymerase of IAVs have been described [109, 110], summarized in Table 1.

Table 1. Selected summary of amino acid changes in avian influenza A virus HA and the polymerase complex associated with adaptation of the virus to the mammalian host

| Protein | Amino acid change/motif | Biological effect |
|---|---|---|
| HA | 323 to 330 (R-X-R/K-R) | Polybasic cleavage motif sequence required for high pathogenicity of H5N1 avian influenza viruses |
| | T156A | Increased virus binding to α2-6 and increased transmission in guinea pigs |
| | G224S | Increased virus binding to α2-6 |
| | Q222L | Increased virus binding to α2-6 |
| | D94N | Increased virus binding to α2-6; enhanced virus fusion |
| | H103Y, T156A, Q222L, G224S | H5 virus transmissible among ferrets |
| | N154D, N220K, Q222L, T315I | H5 HA virus transmissible among ferrets |
| PB2 | L89V | Compensates for the lack of 627K |
| | E158G | Enhances pathogenicity in mice |
| | T271A | Enhances polymerase activity in mammalian cells |
| | G309D, T339K R477G, I495V | Compensate for the lack of 627K |
| | A676T | Compensates for the lack of 627K |
| PB1 | L13P | Observed in human isolates |
| | L472V | L598P Compensate for the lack of PB2-627K |
| NEP | S7L, Y41C ,E75G, X161M | Compensate for the lack of PB2-627K |

### 1.6 Immune response against influenza

IAVs replicate mainly in the epithelial cells of the upper respiratory tract in humans. The respiratory tract through a number of non-specific responses ensures that the host is protected from an IAV infection. These non-specific responses include: the mucin layer and ciliary action. They function to prevent virus entry and uncoating in susceptible cells [6]. However, once these fail and a virus is able to enter, a series of complex responses are stimulated by both the innate and adaptive immune responses. The innate response is rapid and functions mainly to prevent virus replication. Infected cells induce chemokine and cytokine production, which then attract immune cells including macrophages, neutrophils and natural killer (NK) cells [116]. Important cytokines produced include IFN-α/ß which promote the production of antiviral proteins that inhibit protein synthesis, recruit monocytes/macrophages and enhance maturation of antigen presenting cells. Upon experimental infection of humans with IAV, proinflammatory cytokines produced early included IFN-α with IL-6, IL-8 and TNF-α appearing later [117]. High amounts of cytokines produced could lead to high inflammatory responses leading to infiltration of lungs with high amounts of fluids, development of pneumonia and acute respiratory distress syndrome (ARDS) and eventual death. It has been shown in a mouse model that in addition to the high replication rate of the 1918 virus, its severe lethality could also be attributed to infiltration of lungs with neutrophils and alveolar macrophages and severe lung inflammation [118, 119]. This observation could be attributed to the failure of the innate response to suppress viral replication. The failure of the innate response to suppress viral replication could be related to the fact that the NS1 protein which is an IFN-antagonist was efficiently adapted to suppress activation of the IFN system. The adaptive immune response which is activated during this period involves the production of both bone marrow derived lymphocytes (B cells) and thymus-derived lymphocytes (T-cells). B cells are stimulated to produce antibodies specific for the influenza virus subtype. Antibodies are produced against viral proteins HA, NA, NP and M [120]. HA specific antibodies act by blocking the receptor binding site of HA and neutralizing infectivity [121] while NA specific antibodies act by inhibiting virus release from infected cells and preventing further spread [6]. IgA antibodies are secreted onto the mucosal surfaces and provide a first line of defense while IgG antibodies circulate in the blood and are transported to the airways and lungs. Cytotoxic T lymphocytes (CTLs) act by directly lysing infected cells. Antigen presenting cells such as major histocompatibility complexes MHC class I and II present viral peptides to T cell receptors. CTLs recognize infected cells by the expression of MHC complexes on their surfaces and induce lysis of these cells through perforin or granule mediated killing [122]. The adaptive response possesses immunological memory so it is able to store responses for a specific pathogen, so that when the pathogen is encountered again, a faster and more effective response is activated. Influenza vaccination

targets the adaptive immune response to stimulate B and T cells that are capable of rapid responses.

In addition to good health habits such as handwashing with soap, frequent disinfection of surfaces and aversion to strange dead birds, which could help prevent the spread an IAV infection, the best way to prevent an infection is through vaccination [123]. Influenza vaccines are usually made up of virus components of currently circulating viruses. They usually contain influenza A H1N1, H3N2 and an influenza B virus (trivalent influenza vaccine). Quadrivalent vaccines have been manufactured in recent times that contain 2 lineages of influenza B viruses [123]. Although IAV infections are self-limiting, they can be fatal for immunocompromised patients and at risk groups (e.g. pregnant women) and in pandemic situations. There are 3-5 million cases of disease reported worldwide every year with 250 000 to 500 000 deaths despite the availability of vaccines. It is therefore imperative to improve vaccines and medications, make them widely available and convince at risk groups to utilize available vaccines.

### 1.7 Highly pathogenic avian influenza in Ghana

Ghana lies in the coastal belt of West Africa and is bordered by Togo on the East, Cote d'Ivôire on the West, Burkina Faso in the North and the Atlantic Ocean to the south (Figure 6). The country's warm, humid climate has an annual mean temperature between 24°C and 30°C with relative humidity between 77-85%. There are two main seasons in the country; the wet/rainy and the dry/harmattan seasons. The harmattan season lasts from December to March when dry desert air blows into the country from the North. During this time, average temperatures during the day are hotter (up to 39°C), the nights are cooler (as low as 24°C) and the relative humidity is also lowered. The northern part of Ghana experiences a rainy season from April to October. In the South however there are two rainy seasons (April to June) and (September to November). Average rainfall for Ghana is between 78-216mm of rainfall for the year. During the wet/rainy seasons, the country experiences much more frequent and heavier rainfalls (up to 500mm a month). The population of Ghana is 24,658,823 with 38.3% being children between the ages of 0 and 14 years and 4.7% being >65 years. The general life expectancy at birth was estimated at 62.7 years [124].

As discussed in section 1.4, the history of influenza in Ghana dates back to 1891 in colonial Gold Coast. Physicians at the time, recorded unusual number of respiratory related deaths among the population, most likely due to the pandemic influenza of 1889-1893. Therefore when the 1918 pandemic reached the shores of the Gold Coast the disease was not unknown. The Gold Coast was the only country in sub-Saharan Africa at the time that kept

accurate records of transmission, burden and mortalities of the disease. The pandemic is estimated to have killed 1.5-2 million people in sub-Saharan Africa within a very short time and is described as the worst short-term demographic disaster in the history of the continent. In Ghana mortalities were estimated at more than 100 000 in a population of 4 million. Documented evidence shows that Ghana has recorded cases of influenza pandemics that have occurred worldwide [82, 125-127]. In 1973, virological and serological investigations of an influenza-like epidemic in Ghana, confirmed influenza A/Hong Kong/1/68 (H3N2), the 1968 Hong Kong influenza pandemic virus as the causative agent. The estimated attack rate was between 2.5 and 5%. Furthermore, a serological investigation of unusual cases of febrile episodes during the rainy season (June to September) in 1996 found a high prevalence (more than 95% of cases investigated) of H3N2 IAV in Accra [144-146], most likely a second wave of the 1968 Hong Kong influenza pandemic.


**Figure 6. Map of West Africa highlighting Ghana, neighbouring countries and other West African countries.** Ghana is borded by Togo to the East, Burkina Faso to the North, Cote d'Ivôire to the west and the Atlantic Ocean to the south. Modified from google images: blank map of West Africa.

In April 2007, HPAIV of the subtype H5N1 was detected among poultry in Ghana, leading to huge economic losses to farmers and the fear of human infections [88]. In response to this threat, just like many other African countries, the Ghana Health Service and the National Influenza Center (NIC) initiated national surveillance for influenza-like illnesses in collaboration with the WHO, the US-CDC and the US Naval Medical Research Unit No. 3 (NAMRU-3). These surveillance activities have contributed significantly to monitoring of circulating influenza strains and a better understanding of the epidemiology of influenza-like

illness in the country. Through this surveillance system Bonney and colleagues showed that among children <11 years old in Ghana, influenza prevalence was 23% [128]. Because of the fact that there was an existing influenza surveillance network, Ghana was able to detect and respond rapidly to the pandemic of 2009; having laboratory testing and expertise readily available. The surveillance system with support from NAMRU-3, has expanded to include surveillance for influenza viruses among backyard poultry and wild birds living in and around military barracks countrywide. Odoom and colleagues reported in 2012, that no evidence of the presence of avian influenza was found among 680 domestic and wild birds sampled in and around military barracks in Ghana. However, bio-security measures (methods designed to prevent introduction of infections into farms) in these holdings were poor [129]. Studies at live bird markets have also reported poor biosecurity measures and mixture of different birds in the same cage [130]. Again, with support from the US-CDC, various studies have been designed and implemented to look at the burden of influenza disease among high risk groups such as children 0-6 months, pregnant women and HIV patients in a population with continuous demographic surveillance (Shai Osudoku and Ningo - Prampram districts, Accra) in Ghana [131]. Whereas 10 years ago, influenza data from Ghana was scanty, there is currently an appreciable amount of data on influenza in Ghana [132-136].

### 1.8 Aim of the study

Studies on influenza in Ghana have concentrated on surveillance against IAV circulating in the human population. These surveillance activities have contributed significantly to improved influenza monitoring and to better understanding of the epidemiology of influenza-like illness in the country. Other surveillance activities have focused on screening animals (domestic poultry, wild birds, pigs) in and around military barracks in Ghana and in pig farms. Such efforts have made tremendous contributions to the knowledge of the epidemiology of influenza among the poultry population in Ghana. Surveillance activities among domestic poultry, wild birds and in live bird markets reported poor biosecurity measures in these settings [128-130]. These studies therefore concluded that in Ghana, the potential for transmission of infection from birds to humans is considered very high and poses a danger to human health. It was therefore important that studies were initiated that investigated the potential transmission of viruses from avian or swine to humans. Such studies will help us to better understand the ecology of influenza viruses in the aquatic bird reservoir or the swine mixing vessel and perhaps find ways to reduce the burden of catastrophic influenza pandemics. The main aim of this study, therefore, was to investigate the interactions between human and animal influenza viruses in Ghana. The study sought to assess the zoonotic risk posed by avian influenza viruses in Ghana.

# 2.0 Materials

## 2.1 Buffers and solutions

| Buffer | Composition |
|---|---|
| Calcium TBS buffer with 4-MUNANA | 6.8 mM $CaCl_2$ |
| | 0.85% NaCl |
| | 0.02 M TRIS |
| | 40 µM 4-MU-NANA |
| | pH 7.3 |
| Crystal violet solution | 37% formaldehyde in $ddH_2O$ |
| | 1 g crystal violet to 1000 ml |
| EDTA | 10% EDTA in $ddH_2O$ |
| | pH 7.4 |
| PBS (10x) | 26.8 mM KCl |
| | 17.6 mM $KH_2PO_4$ |
| | 1.37 M NaCl |
| | 51.3 mM $Na_2HPO_4$ |
| | $2H_2O$ |
| | Add to 1000ml $ddH_2O$ |
| | pH 7.4 |
| PFA (4%) | 40g PFA in 1x PBS (1000ml) |
| Stop buffer (for 4-MUNANA) | 0.1 M glycine buffer |
| | 25% ethanol |
| | pH 10.7 |
| TAE buffer (50x) | 242g (2M) Tris base |
| | 57.1ml Acetic acid |
| | 100ml 0.5M EDTA pH 8.0 (0.05M) |
| | add to 1000 ml $ddH_2O$ |
| Virus transport medium | 20g Veal Infusion Broth |
| | 4g Bovine Serum Albumin |
| | Add to 1000ml $ddH_2O$ |
| | add 1.6ml gentamicin sulfate, 6.4ml fungizone |
| | sterile filter |

## 2.2 Cells

| Name | Source |
|---|---|
| **Eukaryotic** | |
| Human Embryonic Kidney 293 (HEK 293T: expressing a mutant version of Simian vacuolating SV40 virus large T antigen) | Phillips University of Marburg, Marburg Germany, Prof. dr. Klenk |
| Madin Darby Canine Kidney II (MDCK II) | Phillips University of Marburg, Marburg Germany, Prof. dr. Klenk |
| Human lung cancer cells (Calu-3) | Abt 61 (Gabriel) HPI |

| | |
|---|---|
| Chicken embryonic fibroblasts (DF-1) | Kind donation from Martin Schwemmle, Institute of Virology, University Hospital Freiburg, Germany |
| Human non-small cell lung carcinoma (H1299: p53 deficient) | ATCC (kind donation from the Dobner lab HPI) |
| **Prokaryotic** | |
| E. coli - XL-1 Blue competent cells (Genotype: *recAI endAI gyrA96 thi-I hsdR17 SupE44 relAI lac[*F'*proABlacI*$^{q}$ *ZΔMI5* TN*I0* (Tet$^{r}$)] | Abt 61 (Gabriel) HPI |

**2.3 Chemicals**

| Chemical | Company/source |
|---|---|
| 4-methylumbelliferyl N-acetylneuraminic acid (4-MUNANA) | SIGMA-ALDRICH |
| Agar | SIGMA-ALDRICH |
| Agarose | SERVA |
| Bovine Serum Albumin | SIGMA-ALDRICH |
| Calcium chloride ($CaCl_2$) | SIGMA-ALDRICH |
| Crystal violet | Merck |
| Cytidine-5'-monophospho-N-acetylneuraminic acid sodium salt (CMP) | SIGMA-ALDRICH |
| Dithiothreitol (DTT) | Thermo Fischer Scientific (Kit SuperScript III) |
| Ethanol (EtOH) | Merck |
| Ethidium bromide | Fluka |
| Ethylenediaminetetraacetic acid (EDTA) | SIGMA-ALDRICH |
| Eukitt | Kindler |
| Fungizone | Hyclone |
| Gentamicin sulfate | Gibco Life Technologies |
| Glycerol „UltraPure ™ Glycerol" | Invitrogen |
| Glycine | Geyer Th. |
| Glycogen (*Mytilus edulis*) | SIGMA-ALDRICH |
| Haematoxylin | Shandon |
| Hydrochloric acid (HCl) | Merck |
| Hydrogen peroxide ($H_2O_2$) | Merck |
| Iodine solution (I2), volumetric, 0.5 M I2 (1.0 N) | Fluka |
| Isopropanol (2-propanol) | Fluka |
| Lipofectamin®2000 | Life Technologies |
| Magnesium chloride ($MgCl_2$) | Merck |
| Magnesium sulfate ($MgSO_4$) | Merck |
| Methanol ($CH_4O$) | Chemsolute |
| Ottix Plus | Diapath |
| Ottix Shaper | Diapath |
| Paraffin (low melting) | DCS |
| Paraformaldehyde (PFA) | AppliChem |
| Polyethylenimine (PEI) | Polysciences |

| | |
|---|---|
| Potassium chloride (KCl) | Carl Roth |
| Potassium dihydrogen phosphate ($KH_2PO_4$) | Merck |
| Sodium chloride (NaCl) | Geyer Th. GmbH & Co. KG |
| Veal Infusion Broth | Becton Dickinson |

### 2.4 Consumables

| Item | Manufacturer |
|---|---|
| 6-well plate, cell culture | Corning Incorporated |
| 12-well plates, cell culture | Greiner-Bio-One GmBH |
| 24-well plate, cell culture | Corning Incorporated |
| 96-well plate, cell culture | Sarstedt |
| 96-well plate with V-bottom | NUNC |
| 96-well plate, black, flat bottom | NUNC |
| Craft glue, without solvents | UHU |
| Cell culture flasks T25/T75 with filter cap | Corning Incorporated |
| Cell culture flasks T175 with filter cap | Sarstedt |
| Cell culture dishes 60 mm/10 mm | Corning Incorporated |
| Cryo boxes made of cardboard | Thermo Fischer Scientific |
| Cryotubes, 1 ml and 2 ml | SARSTEDT |
| Disposable Syringes TERUMO® U-100 | TERUMO Corporation |
| EDTA tube 1.5 ml | KABE laboratory technology |
| EDTA capillary blood collection tube (200 µl) | KABE laboratory technology |
| Embedding cassettes for Histology, Micro and Macro | Microm |
| Glass beads for the RETSCH laboratory mill | RETSCH |
| Microcentrifuge tubes 1.5 ml / 2 ml | SARSTEDT |
| Needles (25G x 1 '', 0.5 x 25 mm) | BD Microlance |
| Needles (26 G x 3/8 '', 0.45 x 10 mm) | BD Microlance |
| Neubauer haemocytometre | MARIENFELD |
| PCR tubes /200µl) | SARSTEDT |
| Petri dishes 60 mm / 100 mm | Becton Dickinson |
| Pipette tips, with filter (1-10 µl, 10-100 µl, 100-1000 µl) | SARSTEDT |
| Pipette tips, without filter | Brandt |
| Poly-L-Lysine coated 6well plates | Greiner Bio-one |
| Scalpel, surgical; Cutfix Figure 22 | BROWN |
| SuperFrost / Plus slide | Menzel glasses |
| Transfer pipettes (sterile) 3.5 ml / 10 ml | SARSTEDT |
| Tubes (15ml/50ml) | SARSTEDT |

## 2.5 Enzymes and other reagents

| Enzyme/reagent | Company/source |
|---|---|
| 6x Mass Ruler Loading Dye Solution | Thermo Fischer Scientific |
| BamHI, restriction enzyme | Fermentas |
| dNTPs mix, PCR grade [10mM] | QIAGEN GmbH |
| DpnI, restriction enzyme | Fermentas |
| EcoRI, restriction enzyme | Fermentas |
| HindIII, restriction enzyme | Fermentas |
| KpNI, restriction enzyme | Thermo Fischer Scientific |
| MassRuler DNA Ladder Mix, 80-10 000 bp | Fermentas / Thermo Scientific |
| Neuraminidase from *Vibrio cholerae* | Roche |
| NheI, restriction enzyme | Fermentas |
| RiboLock RI (RNase inhibitor) | Thermo Fischer Scientific |
| α2,3-sialyltransferase from *Pasteurella multocida* | SIGMA-ALDRICH |
| α2,6-sialyltransferase from *Photobacterium damsela* | SIGMA-ALDRICH |

## 2.6 Equipment

| Name | Manufacturer |
|---|---|
| Airflow cabinet "UniProtect" laminar | Zoonlab |
| Autoclave, fixed | Schlumbohm |
| Bath Circulation Thermostat Precitherm | Labora Mannheim |
| Bone shear Bone Shears | Fine science tools |
| Centrifuge 5417R | Eppendorf |
| Centrifuge Avanti J-E | Beckman Coulter |
| Centrifuge Biofuge Pico | Heraeus |
| Centrifuge Multifuge 3S-R | Heraeus |
| Centrifuge Varifuge 3.0R | Heraeus |
| $CO_2$ incubator BBD6220 | Thermo Scientific |
| $CO_2$ Incubator Heracell 150 | Thermo Scientific |
| Digital Camera SZ-10 | Olympus |
| Drying cabinet | Memmert |
| Egg candling lamp | Fritz Gössner |
| Fine scale Extend | Sartorius |
| Gel Documentation System Gel Doc XR | Bio-Rad |
| Gel electrophoresis system Sub-Cell GT | Bio-Rad |
| Ice maker, flake ice | Scotsman |
| Isoflurane Evaporator "Vaporizer" | U.N. |
| Lock autoclave, BSL-3 laboratory | MMM |
| Magnetic stirrer MR3001 | Heidolph |
| Magnetic stirrer MR80 | Heidolph |
| Multichannel pipette (8 channels, 5-50 µl / 20-200 µl) | Brand |
| Microplate reader Tecan Safire2 | Tecan |
| Microplate reader Tristar LB 941 | Berthold Technologies |

| | |
|---|---|
| Microtome HM325 | Microm |
| Microwave oven R-647 | Sharp |
| Mr. Frosty, cryopreservation container | Nalgene |
| PCR machine GeneAmp® PCR System 9700 | Applied Biosystems |
| Paraffin casting station, EG1160 | Leica |
| pH meter 766 Calimatic | Knick |
| Pipettes Eppendorf Reference (1-10 µl, 10-100 µl, 100-1000 µl) | Eppendorf |
| Pipetting aid Pipetus | Hirschmann laboratory equipment |
| Precision balance ED224S | Sartorius |
| Shaking water bath SW-22 | Julabo |
| Spectrophotometer Nanodrop 1000 | Peqlab |
| Standard surgical tweezers | Fine science tools |
| Sterile workbench Herasafe KS 12 | Thermo Scientific |
| Sterile workbench Herasafe KS 18 | Thermo Scientific |
| Surgical fine tweezers | Fine science tools |
| Surgical fine scissors | Fine science tools |
| Thermomixer Comfort | Analytics Jena |
| Transmitted light microscope Axioskop 2 | Zeiss |
| Transmitted light microscope IM | Zeiss |
| Transmitted light microscope Nikon C2+ | Nikon |
| Tissue Infiltration Machine, Vacuum, ASP300 | LEICA |
| Ultrapure water system Milli Q | Millipore |
| Vibrating mill MM400 | Retsch |
| Vortex Mixer 7-2020 | NeoLab |
| Weighing scale for animals | Kern |

## 2.7 For animal experiments and use of animal products

### 2.7.1 Housing and accessories

| Item | Source |
|---|---|
| Female BALB/cJRj mice | Janvier Labs, France |
| IVC cage, type II long | Techniplast |
| Blower unit for IVC frame Blueline | Techniplast |
| Maintenance food, rat / mouse | SSNIFF |
| Water bottle, mice | Techniplast |
| Infusion cap 34 mm, mice | Techniplast |
| Mouse house, red-transparent | Techniplast |

### 2.7.2 Anaesthesia for mice

| Name [concentration] | Manufacturer |
|---|---|
| Isoflurane [100%] | Abbott |
| Ketamine [100 mg/ml] | WDT |
| Sedaxylan (xylazine hydrochloride) [20 mg/ml] | WDT |
| NaCl [0.9%] | B. Braun Melsungen AG |

### 2.7.3 Use of animal products

During this PhD thesis, the following animal products were also used.

1. Embryonated specific pathogen free eggs which were supplied by Valo and incubated at the Forschungstierhaltung (FTH) (animal research husbandry) at the Uniiversität Klinikum Eppendorf (UKE); Hamburg (University Medical Center Hamburg-Eppendorf) for 11 days.
2. Whole blood from chicken (Lohmann Tierzucht ) received in citrate.
3. Whole blood from turkey (Charles Rivers) received in Alsevers solution.

## 2.8 Kits

| Kit | Company/source |
|---|---|
| AgPath-ID One-Step RT-PCR kit | Thermo Fischer Scientific |
| InnuPrep RNA mini kit | Analytik Jena AG |
| Dual Luciferase® Reporter Assay System | Promega |
| Nucleo Bond Maxi Kit | Macherey Nagel |
| Phusion HotStart® II | Thermo Fischer Scientific |
| QIAamp Viral RNA Mini Kit | QIAGEN GmbH |
| QIAprep Spin Miniprep Kit | QIAGEN GmbH |
| QIAquick Gel Extraction Kit | QIAGEN GmbH |
| SuperScript® III | Thermo Fischer Scientific |

## 2.9 Materials for eukaryotic cell culture

| Material | Company/source |
|---|---|
| Albumin solution, bovine (BSA, 35% in DPBS) | SIGMA-ALDRICH |
| Avicel (microcrystalline cellulose) | IMCD GmbH & Co. KG |
| Bovine Serum Albumin (BSA) | SIGMA-ALDRICH |
| Dulbecco's Modified Eagle Medium (DMEM) | SIGMA-ALDRICH |
| Dulbecco's PBS (1x) | SIGMA-ALDRICH |
| Foetal Bovine Serum (FBS) Gold | SIGMA-ALDRICH |
| L-glutamine 200 mM | SIGMA-ALDRICH |
| Minimal Essential Medium (MEM) | SIGMA-ALDRICH |
| Modified Eagle Medium 2X (2X MEM) | SIGMA-ALDRICH |

| | |
|---|---|
| Opti-MEM | Gibco |
| Penicillin and streptomycin (P/S, 100x) | SIGMA-ALDRICH |
| Trypsin-EDTA solution | SIGMA-ALDRICH |
| TPCK-treated Trypsin | SIGMA-ALDRICH |

### 2.10 Media for eukaryotic cell culture

| Medium | Company/source |
|---|---|
| 2X MEM for plaque tests | 2X MEM |
| | 0.4% BSA |
| | 2% L-glutamine |
| Avicel solution | 2.5% Avicel |
| | 25g in 1000ml dd$H_2O$ |
| Cryopreservation medium | FBS |
| | 10% DMSO |
| Growth medium for HEK293T, DF-1,H1299 | DMEM |
| | 10% FBS |
| | 1% L-glutamine |
| | 1% penicillin and streptomycin |
| Growth medium for Calu-3 | DMEM |
| | 15% FBS |
| | 1% L-glutamine |
| | 1% penicillin and streptomycin |
| Growth medium for MDCK II | MEM |
| | 10% FBS |
| | 1% L-glutamine |
| | 1% penicillin and streptomycin |
| Infection medium for MDCK II | MEM 500ml |
| | 0.2% BSA |
| | 1% L-glutamine |
| | 1% penicillin and streptomycin |
| Overlay medium for plaque tests | 50% (2x MEM for plaque tests) |
| | 50% 2.5% Avicel solution |
| Transfection medium for HEK293T | DMEM 500ml |
| | 10% FBS |
| | 1% L-glutamine |

### 2.11 Materials for prokaryotic cell culture

| Medium | Company/source |
|---|---|
| Ampicillin (100 mg/ml) | Serva |
| Bacto Agar | BD Biosciences |
| Glucose | Merck |
| Yeast extract | AppliChem |
| Peptone | AppliChem |
| Tryptone | Sigma-Aldrich |

### 2.12 Media for prokaryotic cell culture

| Medium | Company/source |
|---|---|
| Cryopreservation medium for bacteria | 70% LB-Amp medium |
| | 30% glycerol |
| LB Agar + Ampicillin (100mg/ml) | LB medium |
| | 100mg/ml Ampicillin |
| | 1.5% Bacto agar |
| LB medium + Ampicillin (100mg/ml) | 10 g /1000ml peptone |
| | 5 g /1000ml yeast extract |
| | 10 g /1000ml NaCl |
| | pH 7.5 |
| | 100mg/ml Ampicillin |

### 2.13 Plasmids

| Description | Source / generated by |
|---|---|
| pHW2000-ccdB (with ccdB insert) | Reverse genetics [137] |
| pHW2000-empty vector | Reverse genetics [137] |
| pPolI-NP-Luc (human): Firefly luciferase reporter (*Photinus pyralis*) under human polymerase I promoter. With 3 'and 5' ends of noncoding regions of A / WSN / 33 (H1N1) virus. | Luciferase reporter [111] |
| pPolI-NP-Luc (chicken): Firefly luciferase reporter (*Photinus pyralis*) under a chicken polymerase I promoter | Luciferase reporter [111] |
| pRenilla-TK luciferase reporter (*Renilla reniformis*) | Promega |
| pHW2000-KAN1-PB2-WT | Reverse genetics [137] |
| pHW2000-KAN1-PB1 | Reverse genetics [137] |
| pHW2000-KAN1-PA | Reverse genetics [137] |
| pHW2000-KAN1-NP | Reverse genetics [137] |
| pHW2000-KAN1-HA | Reverse genetics [137] |
| pHW2000-KAN1-NA | Reverse genetics [137] |
| pHW2000-KAN1-M | Reverse genetics [137] |
| pHW2000-KAN1-NS-WT | Reverse genetics [137] |
| pHW2000-R65 PB2 627E | Reverse genetics [137] |
| pHW2000-R65 PB1 | Reverse genetics [137] |
| pHW2000-R65 PA | Reverse genetics [137] |
| pHW2000-R65 HA | Reverse genetics [137] |
| pHW2000-R65 NP | Reverse genetics [137] |
| pHW2000-R65 NA | Reverse genetics [137] |
| pHW2000-R65 M | Reverse genetics [137] |
| pHW2000-R65 NS | Reverse genetics [137] |
| pHW2000-H5N1-10-Acc-PB2 | Reverse genetics [137] |
| pHW2000-H5N1-10-Acc-PB1 | Reverse genetics [137] |
| pHW2000-H5N1-10-Acc-PA | Reverse genetics [137] |

| | |
|---|---|
| pHW2000-H5N1-10-Acc-NP | Reverse genetics [137] |
| pHW2000-H5N1-10-Acc-HA | Reverse genetics [137] |
| pHW2000-H5N1-10-Acc-NA | Reverse genetics [137] |
| pHW2000-H5N1-10-Acc-M | Reverse genetics [137] |
| pHW2000-H5N1-10-Acc-NS | Reverse genetics [137] |
| pHW2000-H5N1-50 Ash-PB2 | Reverse genetics [137] |
| pHW2000-H5N1-50 Ash-PB1 | Reverse genetics [137] |
| pHW2000-H5N1-50-Ash-PA | Reverse genetics [137] |
| pHW2000-H5N1-50-Ash-NP | Reverse genetics [137] |
| pHW2000-H5N1-50-Ash-HA | Reverse genetics [137] |
| pHW2000-H5N1-50-Ash-NA | Reverse genetics [137] |
| pHW2000-H5N1-50-Ash-M | Reverse genetics [137] |
| pHW2000-H5N1-50-Ash-NS | Reverse genetics [137] |
| pHW2000-H5N1-43-Vol-PB2 | Reverse genetics [137] |
| pHW2000-H5N1-43-Vol-PB1 | Reverse genetics [137] |
| pHW2000-H5N1-43-Vol-PA | Reverse genetics [137] |
| pHW2000-H5N1-43-Vol-NP | Reverse genetics [137] |
| pHW2000-H5N1-43-Vol-HA | Reverse genetics [137] |
| pHW2000-H5N1-43-Vol-NA | Reverse genetics [137] |
| pHW2000-H5N1-43-Vol-M | Reverse genetics [137] |
| pHW2000-H5N1-43-Vol-NS | Reverse genetics [137] |
| pHW2000-H5N1-10-Acc-HA_monobasic | Site directed mutagenesis,Reverse genetics [137] |
| pHW2000-H5N1-43-Vol-HA_monobasic | Site directed mutagenesis,Reverse genetics [137] |
| pHW2000-H5N1-50-Ash-HA_monobasic | Site directed mutagenesis,Reverse genetics [137] |
| pHW2000-H5N1-KAN-1-HA_monobasic | Site directed mutagenesis,Reverse genetics [137] |
| pHW2000-H5N1-R65-HA_monobasic | Plasmid kindly generated by Jürgen Stech, FLI, Greifswald - Insel Riems |

### 2.14 Primers

Below is a list of primers that were used for this study. The primers were designed using Clone Manager (v.9) to confirm the presence of HPAIV of the subtype H5N1 from tracheal swab samples. Primers were also designed to sequence large gene products so that the entire gene was covered. Some of the primers used for sequencing were designed by previous laboratory members. Primers were synthesized by Sigma-Aldrich. Previously published primers were used for cloning [137].

**2.14.a Primers used for confirmation of the presence of H5N1**

| Primers | Sequence 5'→3' |
|---|---|
| ChNigH5N1_NP_1226fw | AACCCACTTTCTCGGTACAG |
| ChNigH5N1_NP_1433rev | GAAGGCACGATCGGGTTC |
| ChNigH5N1_NP_1002fw | CTCTGCAGCATTTGAGGAC |
| ChNigH5N1_NP_1207rev | CAGATGCCCTCTGCTG |
| ChNigH5N1_HA_10237fw | GAGGATGGCAGGGAATGG |
| ChNigH5N1_HA_1252rev | GAATCCGTCTTCCATCTTCTTG |
| ChNigH5N1_HA_1456rev | CGTCCCGTTTCTTACACTTTC |
| ChNigH5N1_HA_1236fw | AGATGGAAGACGGATTCCTAG |

fw: forward primer, rev: reverse primer

**2.14.b Sequencing primers**

| Primers | Sequence 5'→3' |
|---|---|
| H5N1_Gh_Seq_PB2_702Fw | CTGCTGGGAGCAAATGTACAC |
| H5N1-PB1-906-Fw | TGCAAATCAGAGGATTCGTG |
| H5N1-Pa-898-Fw | AGGGCAAGCTTTCTCAAATG |
| H5N1_Gh_Seq_HA_693Fw | ATAAACGGGCAAAGTGGCAG |
| H5N1_Gh_NA_Seq_886Fw | ACAATCCACGGCCGAATGAC |
| BGH | TAGAAGGCACAGTCGAGG |
| CMV | CGCAAATGGGCGGTAGGCGTG |

Fw: forward primer

**2.14.c Primers used for cloning**

| Primer name | Sequence 5'→3' |
|---|---|
| PHW-PB2f: | GAAGTTGGGGGGG AGCGAAAGCAGGTC |
| PHW-PB2-2341r: | CCGCCGGGTTATT AGTAGAAACAAGGTCGTTT |
| PHW-PB1-17f: | GAAGTTGGGGGGG AGCGAAAGCAGGCAAAC |
| PHW-PB1-2341r: | CCGCCGGGTTATT AGTAGAAACAAGGCATTT |
| PHW-PAf: | GAAGTTGGGGGGG AGCGAAAGCAGGTAC |
| PHW-PA-2233r: | CCGCCGGGTTATT AGTAGAAACAAGGTACTT |
| PHW-HAf: | GAAGTTGGGGGGG AGCAAAAGCAGGGG |
| PHW-NPf: | GAAGTTGGGGGGG AGCAAAAGCAGGGTA |
| PHW-NPr: | CCGCCGGGTTATT AGTAGAAACAAGGGTATTTTT |
| PHW-N12458f: | GAAGTTGGGGGGG AGCAAAAGCAGGAGT |
| PHW-N12458r: | CCGCCGGGTTATT AGTAGAAACAAGGAGT |
| PHW-Mf: | GAAGTTGGGGGGG AGCAAAAGCAGGTAG |
| PHW-Mr: | CCGCCGGGTTATT AGTAGAAACAAGGTAG |
| PHW-NSf: | GAAGTTGGGGGGG AGCAAAAGCAGGGTG |
| PHW-NSr: | CCGCCGGGTTATT AGTAGAAACAAGGGTG |

### 2.14.d Primers used for site directed mutagenesis

| Primer | Sequence 5'→3' |
|---|---|
| p5_Accra_HA_mono | CAGAAATAGTCCTCAAAGAGAG AGAGGATTGTTTGGAGC |
| p3_Accra_HA_mono | GCTCCAAACAATCCTCT CTCTCTTTGAGGACTATTTCTG |
| p5_Ketu-Obuasi_HA_mono | CAGAAATAGTCCTCAAAGAGAG AGAGGACTGTTTGGAGC |
| p3_Ketu-Obuasi _HA_mono | GCTCCAAACAGTCCTCT CTCTCTTTGAGGACTATTTCTG |
| p5_KAN-1_HA _mono | CAGAAATAGCCCTCAAAGAGAG AGAGGATTATTTGGAGC |
| p3_KAN-1_HA _mono | GCTCCAAATAATCCTCT CTCTCTTTGAGGGCTATTTCTG |

p5: forward primer, p3 reverse primer

### 2.15 Protective gear

| Item description | Manufacturer |
|---|---|
| Biogel gloves | Mölnlycke Health Care |
| Classic Birki | Birkenstock |
| Fabric-reinforced foil tape "Extra Universal" | TESA |
| Laboratory coat | Leiber |
| Laboratory shoes | ABEBA |
| Latex disposable gloves, powder-free | Kimberly-Clark KIMTECH SCIENCE |
| Nitrile gloves, "Purple Nitrile" | Kimberly-Clark KIMTECH SCIENCE |
| OP Hood "Nurse Cap" | Mölnlycke Health Care |
| OP-pants | Sattelmacher |
| Respirator, type 9332 FFP3 valve | 3M |
| Safety goggles | UVEX |
| Surgical mask | Mölnlycke Health Care |
| Super Birki | Birkenstock |
| TYVEK® overboot | DuPont |

### 2.16 Sample collection materials

| Material | Company/source |
|---|---|
| Swabs | Sarstedt |
| Blood collection tubes with clotting activator/serum | Sarstedt |

### 2.17 Softwares

| Program | Source |
|---|---|
| Adobe Design Standard CS6 | Adobe Systems |
| Clone Manager Professional (v.9) | Sci-ed software |
| GraphPad Prism | GraphPad Software |
| Magellan ™ - Data Analysis Software | Tecan Magellan software |
| MEGA (Molecular Evolutionary Genetics Analysis) v 6.0 | Pennsylvania State University |
| Microwin2000 software v.4.41 | Berthold Technologies, software from Mikrotek Laborsysteme GmbH |
| Microsoft Office | Microsoft |
| PyMOL + Tcl-Tk GUI, Molecular Graphics | Schrodinger, LLC |

### 2.18 Viruses

| Name (subtype) | Source/Generated by |
|---|---|
| A/Netherlands/213/2003 (H3N2) | Erasmus University Rotterdam, Rotterdam Netherlands, Dr. med. Thijs Kuiken |
| A/Vietnam/11/1994 (H5N1) HA monobasic made up of recombinant PR8 virus: 7 gene segments of A/Puerto Rico/8/1934 (H1N1) and the HA monobasic of A/Vietnam/11/1994 (H5N1) | Erasmus University Rotterdam, Rotterdam Netherlands, Dr. med. Thijs Kuiken |
| A/chicken/Accra/10/2015 (H5N1) | Reverse genetics, virus rescues [137, 138] |
| A/chicken/Ketu/43/2015 (H5N1) | Reverse genetics, virus rescues [137, 138] |
| A/chicken/Obuasi/50/2015 (H5N1) | Reverse genetics, virus rescues [137, 138] |
| A/Thailand/1 (KAN-1)/2004 (H5N1) | Reverse genetics, virus rescues [137, 138] |
| A/swan/Germany/R65/2006 (H5N1) | Reverse genetics, virus rescues [137, 138] |
| A/Sachsen-Anhalt/101/09 (H1N1) | Kindly provided by Brunhilde Schweiger (Robert-Koch-Institut, Berlin).[139] |
| A/WSN/1933 (H1N1) recombinant virus with HA and NA from A/Aichi/2/1968 (H3N2) | Recombinant virus with 6 internal gene segments of A/WSN/1933 (H1N1) with HA and NA from A/Aichi/2/1968 (H3N2). Kind donation from Eva Böttcher-Friebertshäuser (Phillips University, Marburg) |

# 3.0 Methods

## 3.1 Approvals and Ethical considerations

The study protocol was submitted to the Scientific and Technical Committee and Institutional Review Board of the Noguchi Memorial Institute for Medical Research (NMIMR), College of Health Sciences, University of Ghana, Accra, West Africa and also NMIMR Institutional Animal Care and Use Committee for review and approval. Protocol was approved by the NMIMR Institutional Animal Care and Use Committee (protocol number: 2015-03-2Y, approval dates: from 13.03.2015 – 13.03.2018) and also the Institutional Review Board (IRB: 00001276, approval dates: from 04.03.2015 – 3.03.2016). This protocol was renewed for the period starting 7th May 2017 to 7th April 2018 after a continuous review by the Institutional Review Board of the NMIMR.

All animal experiments were conducted in the safety level 3 laboratories at the Heinrich Pette Institute and were performed in strict accordance with the guidelines of the German animal protection law. All animal protocols were approved by the relevant authority in Hamburg (Behörde für Gesundheit und Verbraucherschutz (Approval number: 82/13).

## 3.2 Sample collection and initial processing

Sample collection sites

Samples for this study were collected from Ghana. Ghana is a relatively small country (area=238,535km²) located in the West African sub-region (section 1.7).

Sampling strategies

In 2014, the poultry population of Ghana was estimated at 68 511 000 and pig population at 682 000 distributed throughout farms nationwide. The strategy was to collect cloacal and tracheal swabs from poultry, nasal swabs and blood from pigs and throat swabs and blood samples from animal handlers. The sampling areas were selected based on the following:

1. Farms and live bird markets in areas/regions that had previous recorded outbreaks of avian influenza A H5N1 (Greater Accra Region, Sunyani in the Brong Ahafo Region and Aflao in the Volta Region). Samples were also collected from animal handlers in the described farms/live bird markets.
2. Small scale farm holdings (less than 300 animals: poultry, duck, guinea fowls and pigs) and animal handlers who agreed to take part in the study.

Based on factors such as access to the farm, availability of animals and farmers on the farm and willingness to participate in the study, a convenient sampling scheme was used in

sampling. The strategy was to collect cloacal and tracheal swabs from more than five birds per trader (if a live bird market was sampled) and more than five birds per farm. Samples were collected from January 2015 to March 2015. During the course of this PhD thesis, an outbreak of HPAIV of the subtype H5N1 was recorded among poultry in Ghana [92]. Samples were also collected from dead chicken from this outbreak in July 2015. In the end, samples collected in total for this PhD thesis are shown in Table 2.

Table 2. List of samples collected in total from Ghana

| Species | Sample type | Number |
|---|---|---|
| Pigs | Nasal swabs, blood | 83 |
| Poultry (chicken, turkey, guinea fowl, duck) | Tracheal swabs, cloacal swabs, blood | 69 |
| Humans (farmers) | Throat swabs, nasal swabs, blood | 27 |
| **Outbreak samples** | | |
| Chicken (Greater Accra Region) | Tracheal and intestinal swabs, lung tissue, brain, spleen | 29 |
| Chicken (Volta Region) | Tracheal and intestinal swabs, lung tissue, brain, spleen | 15 |
| Chicken (Ashanti Region) | Tracheal and intestinal swabs, lung tissue, brain, spleen | 9 |

Sampling

Tracheal swabs, cloacal swabs, nasal and intestinal swabs were collected and placed in vials containing virus transport medium. Blood was collected into blood collection tubes with clotting activator/serum (Sarstedt). Animal samples were always separated from human samples. All samples were kept cool (at 4°C) on the field and transported on ice to the biosafety level 3 (BSL-3) facility of the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Accra Ghana.

Initial sample processing

Human samples were processed at the BSL-2 labs while animal samples were processed at the BSL-3 labs. All blood samples were centrifuged at 2000 g for 10 minutes at 4°C to separate serum. vRNA was isolated from all swab samples using the QIAmp viral RNA isolation kit following the manufacturer's instructions. All swab samples were initially tested in Ghana to determine the presence of influenza virus using a qPCR [140]. All serum samples and samples from the outbreak were shipped to the Heinrich Pette Institute (HPI), Leibniz Institute for Experimental Virology in Hamburg, Germany for further analysis. At the HPI all animal samples were analysed in the BSL-3 labs and human samples at the BSL-2 labs.

### 3.3 Molecular biology methods

#### 3.3.1 RNA isolation

Total RNA was isolated from tracheal swabs collected in virus transport medium using the innuPREP RNA Mini Kit (from Analytik Jena). Starting material for RNA isolation was 140µl of virus transport medium. Total RNA isolation was performed following the manufacturer's instructions. Isolated RNA was stored at -80°C in 5µl aliquots until needed. vRNA was isolated from 900µl of allantois fluids which were positive with the HA assay (3.6.4). vRNA was isolated using the QIAmp viral RNA Mini kit following the manufacturer's instructions. Since the starting sample was 900µl, volumes of downstream buffers were modified accordingly. vRNA was eluted in 30µl of the elution buffer and immediately used or stored at -80°C till further use.

#### 3.3.2 cDNA synthesis

cDNA was synthesized using the Superscript III First Strand synthesis kit in combination with gene specific primers previously designed. Here, the reverse transcriptase in combination with the gene specific primer generates cDNA specific to the gene of interest [designed primers] or a universal primer [137] using deoxy nucleotide triphosphates (dNTPs). The details are given below:

cDNA synthesis

Master mix for one reaction

| Component | 1x reaction |
|---|---|
| RNase-free water | 8µl |
| Primer (10µM) | 2µl |
| dNTP mix (10mM each dNTP) | 1µl |
| Template RNA (2µg) | 2µl |
| Total: | 13µl |

11µl of the mix was aliquoted into PCR tubes and 2µl of template RNA (2µg) added. The tubes were incubated at 65°C for 5minutes in a thermal cycler and then placed on ice for at least 1 minute.

In the meantime, cDNA synthesis mix (Superscript III First Strand Synthesis kit) was prepared as follows:

| Component | 1x reaction |
|---|---|
| 5X Firs-strand Buffer | 4µl |
| 0,1M DTT | 1µl |
| Ribolock RNAse inhibitor 1U (40U/µl) | 1µl |
| SuperScript III ™ Reverse transcriptase (200U/µl) | 1µl |
| Total: | 7µl |

This mixture was added to the RNA + primer mix, re suspended well and incubated in a thermal cycler at: 55°C for 60minutes, 70°C for 15minutes, 4°C to hold.

### 3.3.3 PCRs

#### 3.3.3.1 Amplification of NP to confirm the presence of H5N1 in tracheal swabs

PCR was performed to confirm the presence of NP in tracheal swabs collected from chicken.

H5N1 NP was amplified in the following PCR reaction using Phusion DNA polymerase

| Component | 1x reaction |
|---|---|
| Nuclease free water | 11.4µl |
| 5X Phusion HF buffer | 4µl |
| dNTPs (10mM each) | 0.4µl |
| Primer: ChNig_H5N1_NP_1226Fw (10µM) | 1µl |
| Primer: ChNig_H5N1_NP_1433Rev (10µM) | 1µl |
| Phusion DNA polymerase | 0.2µl |
| cDNA template | 2µl |

The mixture was incubated in a thermal cycler at: 98°C for 30seconds, (initial denaturation), 35 cycles of 98°C for 10 seconds (denaturation), 55°C for 30 seconds (annealing), 72°C for 30 seconds (extension), an additional extension step of 72°C for 10 minutes and a final hold at 4°C.

#### 3.3.3 2 Amplification of influenza viral gene segments for cloning and site directed mutgenesis

All eight viral gene segments were amplified from previously synthesized cDNA (3.3.2) using vRNA (3.3.1) isolated from allantois fluid (3.6.2).

Viral gene segments were amplified in the following PCR reaction using the Phusion DNA polymerase [137]

| Component | 1X reaction |
|---|---|
| Nuclease free water | 29µl |
| 5X Phusion HF/GC buffer | 10µl |
| dNTPs (10mM each) | 1µl |
| Primer forward: (20µM) | 2.5µl |
| Primer reverse: (20µM) | 2.5µl |
| Phusion DNA polymerase | 1µl |
| cDNA template | 4µl |

Amplification was done in a thermal cycler as described (section 3.3.3.1).

### 3.3.3.3 Deletion of multi basic cleavage site of H5N1 HPAIV using site directed mutagenesis

Site directed mutagenesis is used to introduce mutations into plasmid DNA. The mutations could be insertions, deletions or substitutions. In order that H5N1 viruses isolated from Ghana could be handled under BSL-2 conditions, pHW2000 expression constructs for HA with monobasic cleavage sites were generated. In these experiments, the additional arginines in the cleavage site were removed to leave only one arginine which represented a monobasic cleavage site. Complementary primer pairs designed to remove additional arginines were used in a PCR to achieve this. During amplification with the Phusion polymerase, the additional arginines were deleted, leaving one arginine at the cleavage site. Methylated and hemi-methylated plasmids were digested by *DpnI* endonuclease, leaving the mutated plasmid. PCR was performed as shown below using Phusion High Fidelity enzyme:

| Component | 1X reaction |
|---|---|
| Nuclease free water | 33.5µl |
| 5X Phusion HF buffer | 10µl |
| dNTPs (10mM each) | 1µl |
| Primer forward (10µM) | 2µl |
| Primer reverse (10µM) | 2µl |
| Phusion DNA polymerase | 1µl |
| DNA template (50ng) | 1µl |

Mixture was incubated in the thermal cycler at 98°C for 5minutes (initial denaturation), 20 cycles of 98°C for 30 seconds (denaturation), 55°C for 10minutes (annealing), 72°C for 10 seconds (extension) and a final hold at 16°C.

### 3.3.3.4 Cloning of viral gene segments

Cloning was done using previously described primers [137] into pHW2000 expression vectors. Inserted into the pHW2000 expression vectors used was the conserved influenza gene termini and the negative selection marker *ccd*B. The primers were designed such that, each 5' end of the PCR amplicons served as mega primers for the cloning [137]. Because the ends of the PCR amplicons were complementary to the annealing site within the plasmid, it was elongated during a target primed plasmid amplification using a high fidelity enzyme leading to a replacement of the *ccd*B marker by the viral gene. Methylated and hemimethylated forms of the plasmid were digested by the restriction endonuclease *DpnI*. Cloning was done as follows:

Target primed plasmid amplification was performed as described below using Phusion High Fidelity enzyme

| Component | 1X reaction |
|---|---|
| Nuclease free water | 37µl – template DNA |
| 5X Phusion HF buffer | 10µl |
| dNTPs (10mM each) | 1µl |
| 1000ng vector (pHW2000ccdB) | 1µl |
| Phusion DNA polymerase | 1µl |
| DNA template (100ng-300ng) | 1,3,5µl |

Mixture was incubated in the thermal cycler at 98°C for 30 seconds (initial denaturation), 35 cycles of 98°C for 10 seconds (denaturation), 48°C for 1 minute (annealing), 72°C for 5:30 minutes (extension) and a final hold at 4 °C.

PCR products were digested with 1µl of the restriction enzyme *DpnI*. At the end of 60 minutes incubation at 37°C, plasmids carrying the genes of interest were not digested. Plasmids were then transformed into competent *Escherichia coli* (E.*coli* XL-1 Blue cells).

### 3.3.3.5 Agarose gel electrophoresis

Agarose gel electrophoresis analyses are performed to mainly check the success of PCR reactions by checking for the presence of an amplified product of interest. Agarose gels are used to separate amplified DNA by size. Ethidium bromide is added to the agarose because it intercalates into DNA and fluoresces under UV light so that the DNA is visible. A 1% gel is prepared by dissolving 1g agarose in 100ml 1xTAE buffer and heating in the microwave. After it has cooled down to about 40°C, ethidium bromide (10mg/ml) was added and the mixture was poured into an electrophoresis chamber with combs to solidify. When the gels were solid, samples were loaded. Samples (5µl PCR product) were mixed with 6x Mass ruler loading dye solution and loaded. For every gel run, a DNA ladder was always included to help determine the correct sizes of bands. All PCRs performed were analysed on agarose gels run at 100V for 90minutes. Gels were visualized under UV light using the gel electrophoresis and documentation system (Bio-Rad).

### 3.3.3.6 DNA extraction from agarose gels

DNA from PCR products that were used for cloning and site directed mutagenesis were first purified. To do this, all of the PCR products were loaded on the agarose gel and bands of interest excised with a scalpel. DNA was purified from agarose gels using the QIAquick gel extraction kit, following the manufacturer's instructions. DNA was eluted in 30µl of elution buffer.

#### 3.3.3.7 DNA concentration measurement

DNA concentration was determined using the Nanodrop 1000 spectrophotometre. The Nanodrop measures the concentration of both DNA and RNA at absorbance of 260 and 280nm. The ratio of absorbance at 260 to 280 nm (260/280nm) is used to determine the purity of DNA or RNA. A ratio of ~1.8 is considered as “pure” for DNA while a ratio of ~2.0 is considered “pure” for RNA.

#### 3.3.3.8 DNA sequencing

Amplified gene products as well as cloned plasmids were sequenced commercially at Seqlab (Göttingen). Sequences were analysed using Clone manager and in MEGA.

### 3.4 Microbiological methods

#### 3.4.1 Transformation of plasmids into competent E.*coli* bacteria

Plasmids were transformed with DNA into chemically competent E.*coli* bacteria. Competent bacteria stored in 150µl aliquots at -80°C were thawed on ice. Subsequently, 10µl or 20µl of cloning reactions were added and incubated on ice for 30minutes. After incubation, bacteria was subjected to subsequent heat shock at 42°C for 45seconds and cold shock at 4°C for 2minutes. Transformed bacteria were then incubated in 1ml LB medium pre warmed to 37°C without ampicillin for 60minutes at 37°C, shaking at 1000 rpm. Transformed bacteria were spread on LB agar plates containing 100mg/ml ampicillin and incubated at 37°C overnight.

#### 3.4.2 Amplification of plasmid DNA

Plasmid DNA was amplified by growing E. *coli* in LB medium containing ampicillin (100mg/ml). Clones that were picked from the plates the next morning were incubated in 5ml of LB medium containing ampicillin at 37°C for up to 18 hours, shaking at 220 rpm. Large bacteria cultures of up to 250ml of LB medium were also prepared.

#### 3.4.3 Plasmid DNA preparation

Mini preparations

Mini preparations of DNA were prepared from 5ml bacteria cultures grown overnight. Plasmid DNA was isolated from the bacteria using the QIAprep Spin mini prep kit commercially available from QIAGEN.

Maxi preparations

Maxi preparations of DNA were from large cultures (up to 250ml of LB medium) using the Nucleo Bond Maxi kit commercially available from Macherey Nagel. As a modification, in order to increase the yield of the plasmid DNA, 6µg/ml glycogen was added during the isopropanol precipitation stage. The concentration of plasmid DNA isolated from maxi preparations, were adjusted to 1000ng/µl in dd$H_20$. Glycerol stocks of the plasmid were also prepared and stored at -80°C. To prepare a glycerol stock, 300µl of 80% glycerol was added to 700µl of bacteria in LB medium, vortexed and immediately frozen at -80°C.

### 3.4.4 Restriction digest

Restriction digests were performed to investigate whether plasmid DNAs carried genes of interest after cloning and amplification in E.*coli* bacteria. DNA from mini and maxi preparations were digested with various restriction enzymes: BamHI, HindIII, KpNI and NheI according to modified protocols. For all digests prepared, reactions were reduced by 50% in volume. Therefore enzyme concentrations and all other reaction components were halved. The gene map of plasmids were used to determine the correct band sizes. Plasmid DNA sequences were confirmed through sequencing.

## 3.5 Eukaryotic cell culture methods

### 3.5.1 Incubation of eukaryotic cell cultures

All cell cultures and virus cultures were incubated at 37°C, 5% $CO_2$ with 96% relative humidity in $CO_2$ incubators.

### 3.5.2 Maintenance and passage of eukaryotic cells

HEK293T, DF-1 and H1299 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated Foetal Bovine Serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine. MDCK cells were maintained in Minimum Essential Medium (MEM) supplemented as described above, while Calu-3 cells were maintained in DMEM and supplemented as described above but with 15% FBS. Cells were incubated (3.5.1) and cultured up to 80-90% confluency in T-75 cell culture flasks and routinely passaged. To passage cells, all media were pre-warmed to 37°C, cells were first washed with 5ml 1x PBS and incubated (3.5.1) with trypsin-EDTA. Cells were incubated until they detached from each other and from the bottom of the cell culture flask. Trypsin-EDTA was inactivated by re-suspending cells in medium containing FBS. Cells were passaged into fresh culture flasks according to need.

### 3.5.3 Thawing eukaryotic cells

To thaw cells, growth medium was pre-warmed to 37°C. After cells were removed from liquid nitrogen storage, they were thawed immediately at 37°C in a water bath and resuspended in 10ml of growth medium. This was then centrifuged at 1000g for 5minutes to pellet cells. All the supernatant was decanted and cell pellet resuspended in 5ml of growth medium in a T-25 cell culture flask and incubated (3.5.1) overnight. Usually cells were 80-90% confluent the next day. Cells were then further passaged 2-3 times, before they were used in experiments.

## 3.6 Virological methods

### 3.6.1 vRNP reconstitution assay

vRNP activity was determined in human non-small carcinoma cell lines derived from lymph nodes (H1299) and chicken embryo fibroblast cell lines (DF-1), using a dual luciferase reporter activity assay. pHW2000 expression plasmids encoding the viral polymerase genes PB2, PB1, PA and NP were co-transfected with 1µg each of the luciferase-encoding plasmids pPol-I-NP-Luc (Firefly luciferase) but for DF-1 cells pPol-I-NP-Luc (chicken) and for both cell lines pRL-TK (Renilla luciferase). The pPol-I-NP-Luc-human/chicken plasmid encodes the firefly luciferase of the firefly *Photinus pyralis*. The luciferase gene of the plasmid is inserted into the non-coding regions of the influenza A/WSN/33 (H1N1) NP and is controlled by the cellular human/chicken polymerase I promoter (Pol-I), the promoter for the viral RNA-dependent polymerase. When the cells are co-transfected with plasmids encoding the polymerase complex, the viral polymerase complex is artificially reconstructed, leading to transcription and translation of the firefly luciferase. The second reporter luciferase pRL-TK (Renilla luciferase) encodes the luciferase for *Renilla reniformis*, and is under the control of a constitutively expressed herpes simplex virus thymidine kinase (HSV-TK) promoter and therefore served as an internal control for transfection efficiency. Adherent cells: $2.2x10^5$ cells/ml (DF-1) and $2.5x10^5$ cells/ml (H1299) in 12 well plates were transfected using PEI (1µg/µl) and incubated (3.5.1) for 6-8 hours after which medium was changed to a transfection medium. Each experiment was performed in triplicate. Cells were incubated up to 24hours for DF-1 cells and 48 hours for H1299 cells. The substrate for the luciferases was luciferin which released detectable amounts of bioluminescence during cleavage. After incubation, cells were lysed with a 1x passive lysis buffer and luminiscence detected with the Dual-Luciferase Reporter Assay Systems from Promega. The Tristar Multimode Microplate Reader using the Mikrowin2000 v.4.41 software was used [141].

### 3.6.2 Infection of 11 day old embryonated chicken eggs

Specific pathogen free 11 day old embryonating chicken eggs purchased from Valo were infected with lung homogenates from dead chicken. Prior to infection, eggs were inspected for cracks and candled to determine the presence of viable embryos. A viable embryo moved to the light stimulus and also contained visible blood vessels. Eggs which contained no embryos or dead embryos were discarded. The weight of a piece of lung was determined by weighing in tubes containing glass beads. Subsequently, lungs were homogenized in 1ml of 1x PBS in the Retsch tissue homogenizer at 30Hz for 10minutes. After centrifugation at 2000g for 5minutes at 4°C, supernatants were diluted at 1:100 and 1:1000 and used to infect the eggs. Eggs were placed with the blunt ends up because the air sac is present on this side. The shell was disinfected with iodine solution after which a small hole was drilled. With a 1ml syringe (cannula 25G 1 ", 0.5 x 25 mm), 200µl of each lung homogenate dilution was injected into the drilled hole, infecting the allantois cavity. The hole was sealed with glue and after the glue had dried, the eggs were incubated (3.5.1) for 48hours. After 48hours, eggs were chilled at 4°C overnight to contract blood vessels and euthanize living embryos. To harvest the eggs, the shell was perforated at the position where the air sac was located using sterile forceps. After shells and membranes were removed, allantois fluid was harvested with sterile transfer pipettes into 15ml tubes. Allantois fluids were centrifuged at 1000g for 5minutes at 4°C. To determine the presence of virus, HA tests were performed (3.7.3). Viruses with the same HA titres were pooled, re-suspended, aliquoted into cryotubes and stored at -80°C until further use [111].

### 3.6.3 Purification of chicken and turkey blood for haemagglutination assays

Purified erythrocytes from chicken and turkey were used to perform haemaaglutination assays. Whole chicken/turkey blood were washed in 50ml 0.9% NaCl solution in 50ml tubes. After re-suspending in 0.9% NaCl solution, chicken blood was centrifuged at 1000g for 5minutes at 4°C but turkey blood, because the erythrocytes are sensitive were centrifuged at 1000rpm for 5minutes at 4°C. After centrifugation, supernatant consisting of serum, leukocyte and platelet-containing interphase were removed. The washing step was repeated until the supernatant was clear without haemolyzed erythrocytes. Washing steps could be repeated for 3-5 times. After complete removal of the supernatant, a 100% solution was obtained which was diluted to 10% stock solution in 0.9% NaCl solution for further experiments.

### 3.6.4 Haemagglutination assay (HA assay)

The HA assay was used to titrate the amount of haemagglutinating units present in a given sample. This assay takes advantage of the fact that influenza viruses are able to bind to terminally linked sialic acids on the surface of chicken or turkey (or other organisms human, guinea pigs) erythrocytes. When virus is present, HA on the surface of the virus binds to the terminally linked sialic acids on the surface of the erythrocytes to form a lattice: a haemagglutination. In the absence of a haemagglutinating virus, the red blood cells will sink to the bottom and form a button. When the plate is tilted, the button will 'run', forming a characteristic tear drop shape. To perform the HA assay, 2 fold serial dilutions of the sample were performed in V-bottom plates in 1x PBS so that each well eventually contained 50µl of the diluted virus. After dilutions, 50µl of 1% chicken or 0.5% turkey erythrocytes in 0.9% NaCl was added to each well. A negative control of 1x PBS and a positive control (virus of known HA titre) were always included per test. Erythrocytes were allowed to settle for 30minutes at 4°C. The HA titre was the reciprocal of the dilution after which there was no longer a visible haemagglutination. The HA test was suitable for quantitatively determining how many haemagglutination units were present in a given sample, not for how many infectious particles were present. Even if a virus is no longer infectious, the HA on the surface may still be able to agglutinate erythrocyte [142].

### 3.6.5 Haemagglutination inhibition assay (HAI assay)

As previously described (3.6.4), IAV HA binds to sialic acids present on the surface of erythrocytes, leading to the formation of a lattice, agglutination. IAV specific antibodies present in the serum of humans or animals block HA binding sites, leading to a failure of HA to interact with sialic acids. Therefore HA is no longer free to interact with sialic acids on the surface of erythrocytes, leading to abrogation of haemagglutination. By performing serial dilutions, the level of antibodies present in sera can be determined. Sera samples of both humans and animals were first pre-treated to remove residual complement activity by heat treatment; heating sera at 56°C for 30 minutes after which they were diluted 1:10 with 1x PBS. Two-fold serial dilutions of 1:10 diluted sera was prepared in V-bottom shaped 96-well plates in PBS. Viruses were pre-diluted to obtain HA unit of 4/50µl in PBS. Subsequently, 50µl of pre-diluted virus was added to each well containing diluted sera. The plate was incubated for 30 minutes at room temperature after which 1% chicken erythrocytes were added and incubated further for 30minutes at 4°C. A positive HAI test was observed when haemagglutination was not present (blood drips in characteristic tear drop shape) in all wells where the serum contained antibodies. HAI titres were recorded [139, 142].

### 3.6.6 Generation of recombinant influenza A viruses

IAV can be artificially generated or rescued in cell cultures using reverse genetics. This allows for individual virus genes to be manipulated. Reverse genetics rely on the fact that, RNA polymerase I (cellular enzymes that transcribe ribosomal RNA in the nucleus of eukaryotic cells) transcripts are neither polyadenylated nor capped and therefore could represent exact copies of influenza viral RNA. Hoffman and colleagues [138] described methods where both RNA polymerase I transcripts (for vRNA synthesis) and RNA polymerase II transcripts (for mRNA synthesis) are derived from the same template. Viral gene segments (cDNA) are inserted between the RNA polymerase I (pol I) promoter and terminator sequences of pHW200 expression vectors (3.3.3.4). This was further bordered by an RNA polymerase II (pol II) promoter and a polyadenylation site. The orientation of the two transcription units allowed the synthesis of positive-sense mRNA mediated by the cellular polymerase and negative-sense viral RNA. Hence, all molecules from the cellular and viral transcription and translation machineries interacted to produce an infectious IAV. Recombinant H5N1 viruses from Ghana were generated for this study. The pHW2000 expression plasmids (1µg each) encoding cDNA of all 8 viral gene segments were transfected into HEK 293T cells in suspension using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. Transfected cells in 6-well tissue culture plates were incubated for 48hours. MDCK II cells pre cultured in 6-well tissue culture plates were infected with 500µl of supernatant 48hours post transfection and monitored for cytopathic effect (CPE). Successful rescues were confirmed by the presence of CPE and further positive HA tests (3.6.4). Recombinant H5N1 viruses bearing mono basic HA cleavage site (3.3.3.3) were also generated. Recombinant viruses were plaque purified, propagated in MDCK II cells and titrated to determine the concentration using plaque tests [111, 138].

### 3.6.7 Virus propagation in MDCK II cells

MDCK II cells were used to propagate all viruses as well as recombinant viruses generated in this study. MDCK cells were seeded a day before so that cells were 80-90% confluent the next day. Appropriate virus dilutions were prepared in infection medium. Cells were washed with 1x PBS and subsequently infected with infection medium containing virus. This was incubated for 30minutes with shaking of the flask every 10minutes. The inoculum was then removed after incubation and infection medium added. Volumes of virus dilutions and infection medium depended on the flask being used. For T-75 flasks for eg., 5ml of virus dilutions was used for infection with 10ml of infection medium added after incubation and removal of inoculum. When a virus with monobasic cleavage site was being propagated, 1µg/ml TPCK-treated trypsin was added to the infection medium. After infection, flasks were incubated and observed daily for cytopathic effect (CPE). For H5N1 viruses, 70-80% CPE

was observed 24hours post infection, but for H3N2 viruses, CPE was observed up to 72hours post infection. When CPE was observed, virus-containing supernatant was harvested into 50ml tubes and centrifuged at 1000 g at 4°C for 5minutes to remove cell debris. Virus was then aliquoted into cryotubes (100µl to 500µl aliquots), stored at -80°. and thawed only once to use [143].

### 3.6.8 Plaque tests

Virus titres were determined using plaque tests in 24-/6-well tissue culture plates. MDCK cells were seeded in 24-/6-well plates the day before to attain 80-90% confluency overnight. Viruses were then diluted 1:10 to 1:1 000 000 in 1xPBS in 200µl diluents (24-well plates) or 400µl diluents (6-well plates). Cells were washed with 1x PBS and infected with 100µl (24-well plates) or 333µl (6-well-plates) of virus dilutions. Plates were incubated for 30 minutes with shaking every 10minutes. After incubation, 1ml (24-well plates) or 3ml (6-well plates) of a low viscosity overlay medium was added. Plates were then incubated for 72hours without moving. After the incubation period, low-viscosity overlay medium was removed and plates washed with 1x PBS. Virus replication was stopped by fixing cells with 0.5ml (24-well plate) or 1ml (6-well plate) 4% PFA for at least 30minutes at 4°C. Virus plaques were visualized by removing PFA and flooding the cell layer with crystal violet solution. This stained viable cells, leaving plaques as holes in the cell layer which could be clearly seen and counted. Virus titres are then calculated from counted plaques as plaque forming units/ml [143, 144].

### 3.6.9 Plaque purification of recombinant viruses

Recombinant viruses were plaque purified using the 6-well format for plaque tests (3.6.8). After 30minutes incubation with shaking every 10minutes in between incubation, instead of a low viscosity overlay medium, a 1:1 ratio of 1.8% cool agar solution and 2xMEM was added. After agar was set, plates were incubated for 72hours. Single plaques were excised from agar using 1000µl filter tips with the pointed tips cut off. Agar was then incubated in 200µl of 0.2% BSA in 1x PBS at 4°C for 72hours after which supernatant was used to infect MDCK cells in T-25 flasks for P1 virus stock generation. P2 virus stocks were generated and titrated for use in animal experiments.

### 3.6.10 Thermal stability determination

To determine the thermal stability of H5N1 HPAIVs, viruses were exposed to temperatures of 50°C after, which amount of infectious virus particles left were determined. For each virus, 200µl aliquots in 1.5ml tubes were incubated at 50°C. Tubes were removed at 60minute intervals for up to 360minutes and kept at 4°C overnight. The amount of infectious virus particles left was determined using plaque tests in 6-well plate format (3.6.8)[144].

### 3.6.11 Receptor specificity determination

In order to determine cell surface receptors that H5N1 viruses preferred binding to, HA assay with modified turkey red blood cells was performed. Virus HA titres were first adjusted to 64HAU. After turkey blood had been washed (3.6.3), sialic acids were completely removed from the surface of the erythrocytes by incubation with 50mU of *Vibrio cholera*e neuraminidase with 8mM $CaCl_2$ solution at 37°C for 60minutes, re-suspending every 20minutes. De-sialylated turkey erythrocytes were resialylated by addition of 6mU α2,3-sialyltransferase from *Pasteurella multocida* or 38mU of α2,6- sialyltransferase from *Photobacterium damsela* in the presence of 30mM CMP and incubated at 37°C for 120minutes, re-suspending every 20 minutes. After the incubation period, red blood cells were washed up to 3 times with 1x PBS solution containing 1% BSA. HA tests were then performed using the modified turkey erythrocytes [145].

### 3.6.12 Neuraminidase activity determination

To determine the neuraminidase activity of H5N1 viruses, the fluorescent compound 4-methylumbelliferyl N-acetylneuraminic acid (4-MUNANA) was used. NA can cleave 4-MUNANA into 4-MU (4-methylumbelliferone), which fluoresces. Fluorescence could then be measured. The NA activity therefore was directly proportional to amount of fluorescence. Each virus was diluted to 8HAU after, which 5µl was incubated with 50µl of 40mM 4-MUNANA solution in calcium TBS buffer in black 96-well plates. Plates were incubated for 30 minutes. The reaction was stopped with 100µl of stop buffer (0.1M glycine buffer in 25% ethanol). Fluorescence of 4-MU was detected in Tecan Safire 2 at λ excitation = 355 nm and λ emission = 460 nm [146].

### 3.6.13 Growth kinetics

To determine how well H5N1 viruses replicated in cells, viral growth kinetics were performed. Chicken embryo fibroblasts (DF-1) seeded at 3.0 $x10^5$cells/ml and human airway epithelia cells (Calu-3) seeded at 4.0 $x10^5$cells/ml were infected with 500µl of viruses at MOI of 0.001 6-well plates. Infection experiments were performed in triplicates. Plates were incubated for 30minutes shaking every 10minutes. After incubation, the inoculum was removed and infected cells were washed up to 3 times with 1ml of infection medium after which, 3ml of infection medium/well was added. An aliquot of 200µl of supernatant was immediately frozen at -80°C to represent time point zero. Supernatants (200µl) were aliquoted during 24-hourly intervals for up to 96hours. Supernatants were frozen at -80°C and thawed once to determine virus titres with plaque tests in 24-well plates (3.6.8) [111].

### 3.7 Animal experiments

To determine the virulence of H5N1 HPAIV Ghanaian isolates, 8 weeks old female BALB/cJRj (Janvier Labs) mice were infected.

#### 3.7.1 Narcosis of female BALB/c mice

Mice were anaesthesized before infection. The mice were placed in an inhalation chamber and sedated with isoflurane (gas). After sedation, they were injected intraperitoneally using a 1ml syringe and a 26G cannula with a mixture of 100mg/kg ketamine and 20mg/kg xylazine in 200µl 0.9% NaCl. The amount of anaesthesia depended on the weight of the mouse. When mice needed to be killed, after sedation with isoflurane, cervical dislocation, the most humane way of euthanizing the mice was done. When blood was collected from mice, they were also pre-sedated with isoflurane gas.

#### 3.7.2 Intranasal infection of mice for weight loss, survival and organ harvesting

Mice were intranasally infected with 50µl of $10^2$p.f.u./ml of H5N1 HPAIV isolates as well as 1x PBS for PBS control animals. For infection, 50µl of pre-diluted virus or PBS, was administered slowly into the nostrils of the mice with a pipette. After infection, mice were placed with their heads in an upright position to ensure the inoculum did not leak out. Respiratory rates of mice were also monitored. The mice were observed and weighed daily for 14 days for weight loss and survival. Weights were documented every day and the mice were scored on their appearance and behaviour as well. Mice were euthanized when they had lost >25% of their starting body weight and appeared lethargic, unable to eat or drink, showed visible signs of infection (shivering) or experienced obvious pain. The Reed and Muench method [147] was used to determine the dose at which the mice died had to be euthanized ($MLD_{50}$). A percentage of infected mice (3 mice/virus or PBS) were sacrificed 3 days and 6 days post infection and lungs harvested. Blood was first drawn from the retro bulbar into EDTA tubes before lungs were harvested. Blood was centrifuged at 2000 g for 10minutes to separate serum which was aliquoted and stored at -80°C. The mice were euthanized by cervical dislocation and the abdominal and thoracic cavities opened up. Lungs were then subsequently removed. Half of each lung was fixed in 10ml 4% PFA solution for histology and the remaining half, minced and stored in vials containing glass beads. The weight of these lungs were determined and frozen at -80°C. To determine virus titres in lungs of infected mice, frozen lungs were thawed once and homogenized in 1ml 1x PBS in tubes containing glass beads in the Retsch mill at 30Hz for 10minutes. Tubes were then centrifuged at 1 000 g for 5minutes at 4°C. Supernatants were used directly in plaque tests in 6-well plate formats (3.6.8) [148].

### 3.8 Histology

#### 3.8.1 Haematoxylin and Eosin (H&E) staining

Lung tissue samples were stained to determine effect of H5N1 HPAIV infection in the lungs of mice. The H&E staining is mainly to differentiate between the nucleus and cytoplasm of cells. Haematoxylin is a dark blue or violet stain that is basic/positive. It therefore binds to basophilic substances (DNA/RNA present in nucleus, which is acidic and negatively charged) and stains them violet. Eosin is a red or pink stain that is acidic/negative. It binds to acidophilic substances such as positively charged amino acid side chains (e.g. lysine, arginine) abundantly present in cytoplasm, staining the cytoplasm reddish. The H&E staining was performed as previously described [148]. Prior to staining, the following were performed:

Preparation of slides

Lungs were stored in 4% PFA for minimum of 2 weeks after which slides were prepared from formalin-fixed lung tissues for histology. Lung tissues were first dehydrated by placing in increasing concentrations of ethanol (listed below) to remove water and formalin. Ethanol was subsequently removed by addition of xylene. Lungs were then embedded in paraffin blocks. Paraffin blocks were cooled down to -12°C for 30minutes. The HM325 microtome was used to cut 4µm thick sections which were mounted on slides and dried overnight at 37°C.

**Dehydration and infiltration**

1. Ethanol 70 %, 1 hour
2. Ethanol 80 %, 1 hour
3. Ethanol 90 %, 1 hour
4. Ethanol 95 %, 1 hour
5. Ethanol 100 %, 1 hour
6. Ethanol 100 %, 1.5 hours
7. Xylene I, 1 hour
8. Xylene II, 1 hour
9. Paraffin type 3, 58 °C, 1 hour
10. Paraffin type 3, 58° C, 1 hour
11. Paraffin type 3, 58 °C, 1 hour

Tissue was then deparaffinzed and rehydrated in the following steps:

1. Xylene, 3 x 5 min
2. Ethanol 100 %, 5 min
3. Ethanol 70 %, 5 min
4. Ethanol 40 %, 1 min
5. Storage of slides in water

H&E procedure:

1. Hematoxylin solution, 4 min
2. $ddH_2O$, 10 seconds
3. Tap water washing, 4 minutes
4. Eosin G-solution 1 %, 30 seconds
5. 3x ddH2O, 15 seconds
6. 2x ethanol 70 %, 15 seconds
7. 2x ethanol 90 %, 15 seconds
8. 2x ethanol 100 %, 15 seconds
9. 3x xylene, 5 minutes

### 3.9 Statistical analysis

All data were analyzed with GraphPad Prism 5.03 software. The Reed and Muench method was used to determine the dose at which 50% of the mice died ($MLD_{50}$). Unpaired t-tests and ANOVA were used to calculate significances between and among viruses. Statistical significance was defined as $p<0.05$ (*$p<0.05$; ** $p<0.01$; *** $p<0.001$) and stated in each figure legend.

## 4.0 Results

In Ghana, farmers are usually not fully protected when handling animals like pigs and chicken. Many people operate backyard poultry farms and small pig holdings, which places humans in close proximity with these animals. Studies on IAVs in Ghana however, have either concentrated on the activity of the virus in the human or animal populations. This thesis aimed to study IAVs at the human/animal interface due to the potential danger posed by the closeness of humans to animals such as chicken and pigs. Therefore we initially performed a sero-prevalence study, to investigate occurrence of IAV among Ghanaian farmers and animals. Through the course of this PhD studies, there was an outbreak of HPAIV of the subtype H5N1 among poultry in West Africa, which spilled over into several West African countries including Ghana. Viruses isolated from this outbreak were analyzed for their ability to transmit to humans.

### 4.1 Influenza sero-prevalence among farmers and animals in Ghana

In this study, we recruited farmers from selected farms in the Greater Accra Region of Ghana. A total of 27 farmers involved in poultry and pig farming in the Greater Accra Region of Ghana were enrolled into the study. A map of Ghana is shown in Figure 7 displaying sample collection areas. Selected farms and live bird markets where farmers were enrolled are further depicted in Figure 8. Enrolled farmers were 18-79 year old with 96.3% (26/27) being male (Table 3). Majority of the farmers, 55.6% (15/27) were pig farmers, having worked with pigs for periods ranging from 1-30 years. Poultry farmers consisted of 29.6% (8/27), while farmers who worked with a mixture of pigs and poultry comprised 14.8% (4/27). All farmers (100%), across all age groups exhibited antibodies to the HA of influenza A/Aichi/2/1968 (H3N2) with the internal genes of A/WSN/33 virus (2.18) to protective levels (HAI titres: ≥ 1:40) except for 10% of 18-29 year olds who showed antibody levels <1:20 (Figure 9A). Up to 50% of 18-29 year old farmers possessed antibodies to A/Netherlands/213/2003 (H3N2) to protective levels (Figure 9B) compared to farmers aged 30-49 years, 33.3% of whom displayed protective antibody levels. Protective antibody levels were also recorded for 14.3% of 50-59 year olds but not for 60-79 year old farmers. Older farmers 40-79 year olds presented protective antibody levels (66.7%: 40-49 year old, 14.3%: 50-59%, 100%: 60-79 year olds) to A/Sachsen-Anhalt/101/2009 (H1N1) (Figure 9C), representing the 2009 influenza pandemic virus in comparison to younger farmers 18-39 year olds who presented antibody titres of <1:20. All farmers showed no antibodies to A/swan/Germany/R65/2006 (H5N1). Farmers were assessed for occupational risk factors for aquring or transmitting influenza infection from or to animals (Figure 9D). It was observed that all farmers (100%) never washed their hands prior to handling the animals nor wore facemasks while handling animals. Close to 80% always wore gloves and 63% wore boots

when working with animals. Up to 96% of farmers never wore aprons when working with animals, but all (100%) farmers washed their hands after handling animals. A total of 152 samples were collected from animals, 55% of were from pigs (Figure 9E) with the remainder from poultry (chicken, turkey, guinea fowl, ducks). All animal samples were tested for antibodies to A/Netherlands/213/2003 (H3N2), HA of influenza A/Aichi/2/1968 (H3N2) present on WSN/33 virus (2.18), A/Sachsen-Anhalt/101/2009 (H1N1) as well as A/swan/Germany/R65/2006 (H5N1) viruses. No antibodies to any of the IAVs were detected in animal sera.

Altogether, we could show that all Ghanaian farmers demonstrated antibodies to the HA of influenza A/Aichi/2/1968 (H3N2) with the internal genes of A/WSN/33 virus. Up to 50% of young farmers (18-29year olds) possesesed antibodies to a more contemporary H3N2 IAV strain. Older farmers (40-79 year olds) exhibited protective antibody levels to the 2009 pandemic IAV. We could further show after analysis of risk factors associated with transmission that Ghanaian farmers were at high risk for zoonotic events. We observed that farm animals in Ghana, did not exhibit antibodies to IAVs that we tested against, an indication that there was no transmission from farmers to animals at the time of the study.

**Map of Ghana showing administrative regions**

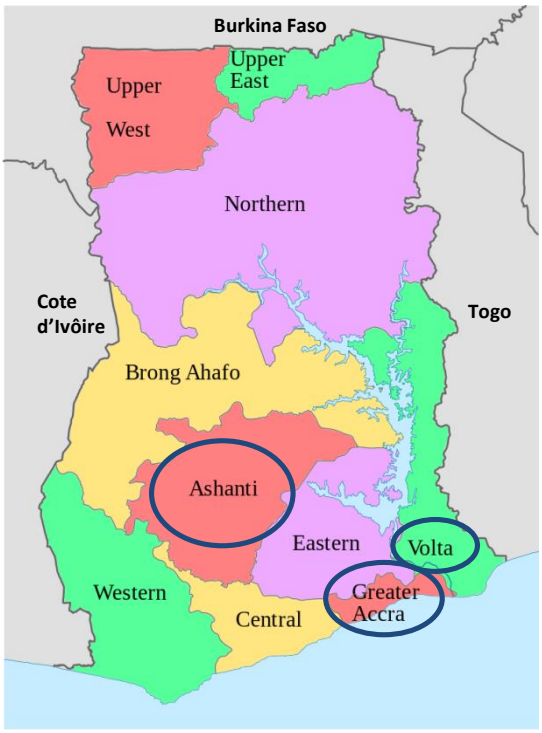


**Figure 7. Administrative map of Ghana**. Different regions are shown in different colours. Sample collection areas are highlighted with dark blue circles. Modified from google images: map of Ghana showing administrative regions.

Table 3. Age distribution of farmers from selected farms in Accra, Ghana (n=27)

| Variable<br><br>Age (years) | Poultry farmers n= 8 (29.6%) | Pig farmers n= 15 (55.6%) | Farmers with poultry and pigs n= 4 (14.8%) |
|---|---|---|---|
| 18-29 | 5 (18.5%) | 5 (18.5) | 0 (0%) |
| 30-39 | 1 (3.7%) | 2 (7.4%) | 3 (11.1%) |
| 40-49 | 1 (3.7%) | 2 (7.4%) | 0 (0%) |
| 50-59 | 1 (3.7%) | 6 (22.2%) | 0 (0%) |
| 60-79 | 0 (0%) | 0 (0%) | 1(3.7%) |
| | | | |
| How long farmer has worked with animals (years) | | | |
| 1-10 | 6 (22.2%) | 8 (29.6%) | 4 (14.8%) |
| 11-20 | 1 (3.7%) | 5 (18.5%) | 0 (0%) |
| 21-30 | 1 (3.7%) | 2 (7.4%) | 0 (0%) |


**Figure 8. Pictures showing selected farms and live bird markets in the Greater Accra region where samples were collected**. A: backyard farm <300 animals with a mixture of poultry, ducks and turkeys in one pen B: pig houses which are designed such that birds have access to the pigs C: live bird market in Accra (the capital of Ghana) where birds are prepared on site (red arrow) D: commercial farm >1000 birds/holding with enclosed houses.

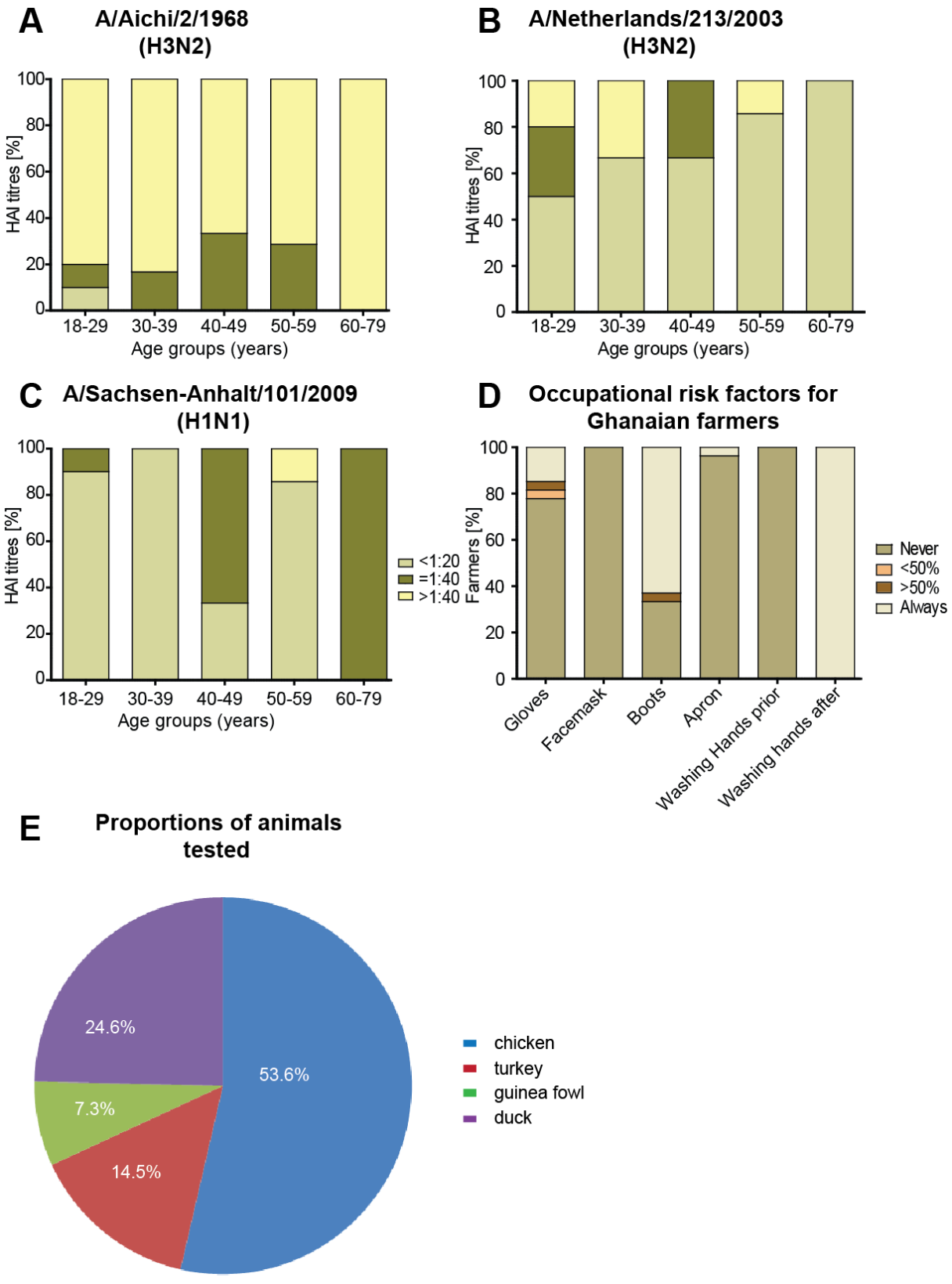


**Figure 9. Influenza sero prevalence and occupational risk factors among animal handlers from selected farms in the Greater Accra Region of Ghana**. Animal and human sera were tested in HAIs against: **A**. A/Aichi/2/68 (H3N2) an old strain, **B**. A /Netherlands/213/03 (H3N2), a contemporary strain, **C**. A/Sachsen-Anhalt/101/09 (H1N1) representing the 2009 influenza A pandemic strain. **D**. Occupational risk factors among the farmers were assessed and **E.** Proportions of animal samples tested.

### 4.2 Characterization of H5N1 HPAIV isolates from chicken in Ghana

During the course of this PhD thesis, an outbreak of H5N1 HPAIV among chicken in Ghana was recorded. This outbreak started in Nigeria and spread throughout the West African sub-region and into Ghana, leading to the death and culling of approximately 1 000 000 chicken in the sub-region. For this thesis, tracheal swabs, lung tissue samples among other samples were collected from dead chicken. Isolated H5N1 HPAIV were analysed *in vitro* and in *vivo* for their zoonotic potential.

#### 4.2.1 Screening of samples for viral nucleoprotein

Animal samples were tested for H5N1 HPAIV by PCR. Tracheal swab specimen collected from dead chicken samples were screened for viral NP. This was done in a two-step PCR to using cDNA previously synthesised from isolated total RNA. Primers were designed to target NP and in combination with a high fidelity DNA polymerase, amplified a region which was highly conserved in NP (product size of 208bp). A sample gel photograph from the RT-PCR of tracheal swab specimens collected from the Greater Accra Region is shown in Figure 10. Out of a total of 29 samples tested for the Greater Accra Region, 70% (20/29 tracheal swabs) were confirmed as positive for H5N1, the rest were negative. For samples from the Volta region (15 tracheal swabs) and the Ashanti region (9 tracheal swabs), all samples were confirmed as positive for NP of H5N1 HPAIV.


**Figure 10. Agarose gel photograph showing PCR products consistent for the presence of NP from chicken tracheal swab specimens from the Greater Accra Region of Ghana**. A 2.5% agarose gel was run after a two-step RT-PCR to detect the presence of H5N1 HPAIV NP from tracheal swabs. Expected product size was 208bp. Shown here is a section of a gel photograph of samples from the Greater Accra Region. M=100bp molecular weight marker (Gene Ruler, Fermentas), PCR positive control was confirmed influenza A H5N1 positive cDNA (A/swan/Germany/R65/2006 (H5N1)). Arrow indicates NP band size (approximately 208bp). Lanes: 1:Negative control (cDNA without reverse transcriptase), 2:Negative control (PCR master mix alone), 3-19:Tracheal swab samples from Greater Accra Region (1-17) and 20:PCR positive control.

### 4.2.2 Phylogenetic and genetic analysis of H5N1 HPAIV isolates

For phylogenetic analysis, virus was first propagated in embryonated chicken eggs from lung tissues already obtained. Then viral RNA was isolated and sequenced for HA, NA, PB2 and NP genes. Phylogenetic analysis of HA, NA, PB2 and NP showed that, viruses isolated from Ghana belonged to the H5N1 virus clade 2.3.2.1c (Figure 11). Viruses belonging to this clade are re-assortant viruses containing PB2 of H9N2 viruses. The PB2 of Ghanaian virus strains, clustered with H9N2 viruses isolated in Asia during 2007-2013, confirming that, PB2 really belonged to H9N2 viruses. All viruses from Ghana clustered with contemporary virus strains from Europe, Asia, the Middle East and other West African countries (i.e., Burkina Faso, Côte d'Ivoire, Niger and Nigeria) and were closely related to the H5N1 outbreak strain from Nigeria: A/chicken/Nigeria/15/VIR339-2/2015 (Figure 11). Genetic analysis of sequenced viral proteins showed that, strains from Ghana exhibited 80-98% homology with the outbreak strain from Nigeria. Genetic analysis of HA confirmedthe presence of a multi basic cleavage site (RERRRKR/GLF), which is common for H5N1 HPAIVs. When compared with the outbreak strain from Nigeria, HA from Ghanaian isolates differed by 9 amino acids. The HA from all Ghanaian strains contained several human adaptive mutations e.g. D94N, S133A, S155N, T156A which had been associated with increased virus binding to human-type SAα2,6Gal (Table 4). The NA from Ghanaian isolates differed from the Nigerian outbreak strain by 10 amino acids and did not harbour any drug resistance mutations. The PB2 from Ghanaian viruses although lacked the E672K and D701N human adaptive signatures, possessed 5 out of a group of 7 mutations which had been shown to enhance polymerase activity and increase virulence in mice. The NP from Ghanaian virus strains showed 99% homology with the outbreak strain from Nigeria and differed by 2 amino acids to the Nigerian isolate. This further confirmed the possibility that the virus was imported into Ghana from Nigeria. The NP lacked the human adaptive signature N319K. These data are published in the Emerging Infectious Diseases: Asante *et al.,2016* [92]. A comparison among H5N1 HPAIV Ghanaian isolates as well as with control viruses R65 and KAN-1 is shown in Table 5. In HA H5N1 HPAIV Ghanaian isolates differed from each other by 6 amino acids, in NA by 6 amino acids, in PB2 by 4 and in NP by 1 amino acid. These observations demonstrate that H5N1 HPAIV Ghanaian isolates show high homology to each other. In Table 5, we show amino acid motif changes in Ghanaian isolates similar to KAN-1, a human H5N1 HPAIV isolate. Out of a total of 34 amino acid changes observed in HA when Ghanaian isolates were compared with R65 and KAN-1, approximately 18%, were similari to KAN-1. H5N1 HPAIV belonging to clade 2.3.2.1c have previously caused deaths in humans, therefore the potential risk for transmission from infected poultry to humans was a major concern. Due to this, viruses were additionally analysed for their zoonotic potential.

In summary, we could show that H5N1 HPAIV that caused an outbreak among poultry in Ghana in 2015, belonged to the H5N1 clade 2.3.2.1c, showing high homology to H5N1 virus strain that caused outbreaks in Nigeria in January 2015. Our virus strains harboured several significant mutations in viral proteins HA, NA, PB2 and NP, which had been described to be involved in the adaptation of avian IAVs to mammalian hosts. We could further show that H5N1 HPAIV Ghanaian isolates, remained highly similar to each other and harboured mutations observed in KAN-1, H5N1 HPAIV human isolate.

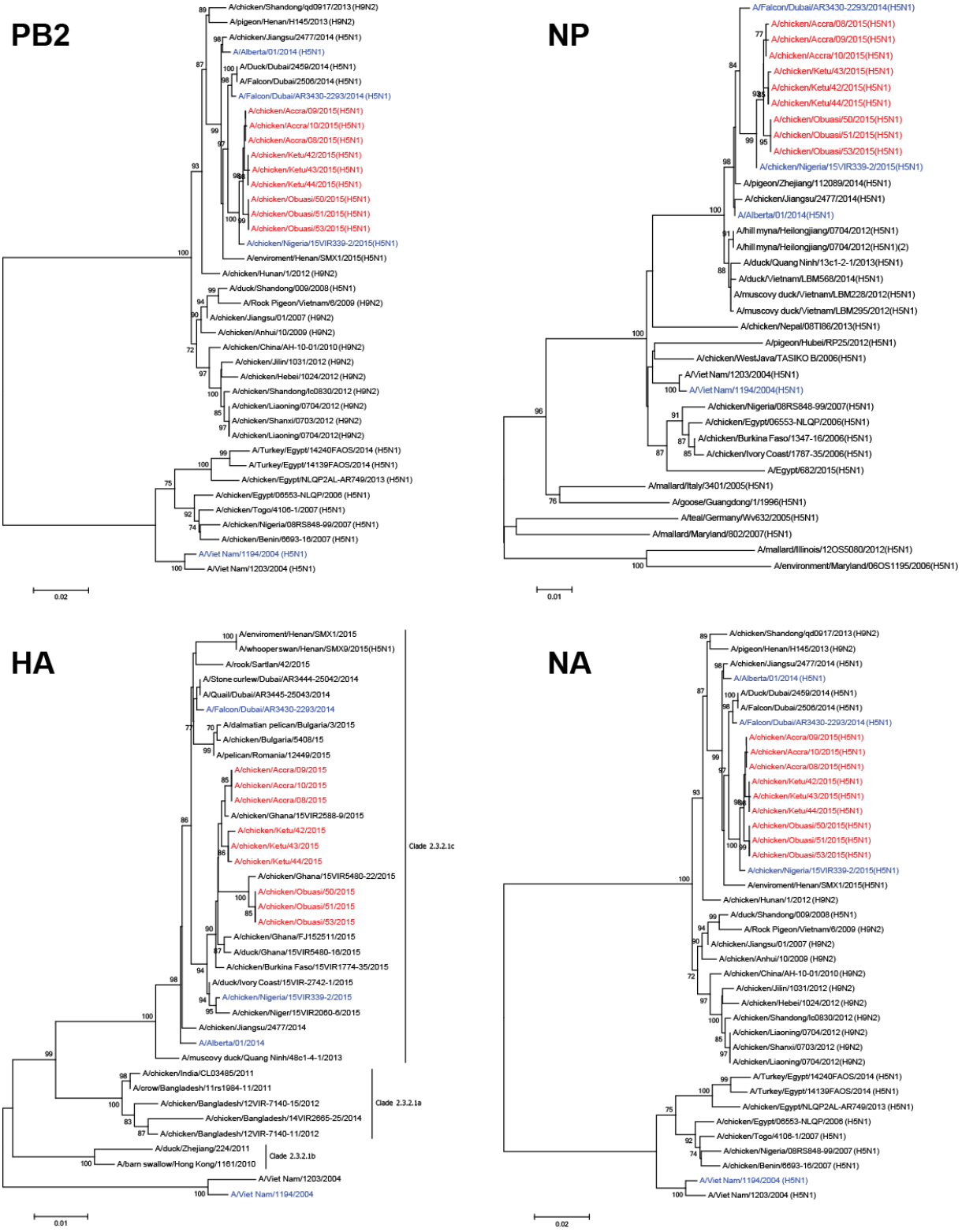


**Figure 11. Phylogenetic analysis of selected proteins of H5N1 HPAIV isolates from chicken in Ghana in 2015**. Viruses sequenced from this study are shown in red and reference viruses are in blue; other sequences were downloaded from the Global Initiative on Sharing Avian Influenza Data (http://platform.gisaid.org) and GenBank databases. Evolutionary analyses were conducted with MEGA6 (http://www.megasoftware.net/). Bootstrap values >70% of 500 replicates are shown at the nodes. The scale bar signifies the number of nucleotide substitutions per site.

Table 4. Mutations observed for Ghanaian viruses compared with global strains as well as the 2015 outbreak strain from Nigeria A/chicken/Nigeria/15VIR339-2/2015**.**

| Protein | Region | Mutation | Function | Literature |
|---|---|---|---|---|
| Basic Polymerase protein 2 PB2 | All regions | **L89V, G309D, T339K, R477G,** I495V, **K627E,** A676T | Enhanced polymerase activity, increased virulence in mice | [149] |
| | | I495A, A676M | | |
| | | M464L, V511I | unknown | |
| | Accra, Ketu | M295V | | |
| | Ketu | R17C | | |
| | Obuasi | K197R | | |
| Haemagglutinin HA | All regions | D94N | Increased virus binding to α2-6; enhanced virus fusion | [150] |
| | | S133A | Increased pseudovirus binding to α2-6 | [151] |
| | | S155N | Increased virus binding to α2-6 | [152] |
| | | T156A | Increased virus binding to α2-6 and increased transmission in guinea pigs | [152, 153] |
| | | S155N, T156A | Increased virus binding to α2-6 | [152, 153] |
| | | 323 to 330 (RERRRKRG) | Polybasic cleavage motif sequence required for high pathogenicity of H5N1 avian influenza viruses | [108, 154-157] |
| | All regions | T235P, I377V, K397R | unknown | |
| | Accra | S356R | | |
| | Ketu | T71S (G163S, T188A)* | | |
| | Obuasi | K259R, K372R, N475D, M478I | unknown | |
| Nucleoprotein NP | All regions | S450N | unknown | |
| | Obuasi | Q398L | | |
| Neuraminidase NA | All regions | E99Q | unknown | |
| | Accra | I74V, R410Q | | |
| | Accra, Ketu | S319L, S365C | | |
| | Ketu | V243I, S430G | | |
| | Obuasi | I143T, N202D, S319F | | |

Bold fonts: 5 mutations observed in sequences from Ghana which are part of a group of 7 mutations which have been published to enhance polymerase activity and increase virulence in mice [171]. *Mutations in bracket were present in one out of 3 samples from Ketu [92].

Table 5. Comparison among H5N1 highly pathogenic avian influenza Ghanaian isolates and with control viruses R65 and KAN-1

| | **Haemagglutinin** | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Amino acid positions** | | | | | | | | | | | | |
| Virus | 83 | 129 | 189 | 252 | 386 | 472 | | 71 | 259 | 356 | 372 | 475 | 478 |
| A/swan/R65/Germany/2006 | I | S | R | N | N | R | | | | | | | |
| A/Thailand/1(KAN-1)/2004 | A | L | K | Y | D | K | | | | | | | |
| A/chicken/Accra/10/2015 | A | L | K | Y | D | K | | T | K | R | K | N | M |
| A/chicken/Ketu/43/2015 | A | L | K | Y | D | K | | S | K | S | K | N | M |
| A/chicken/Obuasi/50/2015 | A | L | K | Y | D | K | | T | R | S | R | D | I |
| | **Neuraminidase** | | | | | | | | | | | | |
| Virus | 44 | 53 | 91 | 365 | | 74 | 143 | 202 | 243 | 319 | 365 | 410 | 430 |
| A/swan/R65/Germany/2006 | R | K | R | S | | | | | | | | | |
| A/Thailand/1(KAN-1)/2004 | H | N | K | S | | | | | | | | | |
| A/chicken/Accra/10/2015 | H | N | K | C | | V | I | N | V | L | C | Q | S |
| A/chicken/Ketu/43/2015 | H | N | K | C | | I | I | N | I | L | C | R | G |
| A/chicken/Obuasi/50/2015 | H | N | K | S | | I | T | D | V | F | S | R | S |
| | **Polymerase basic protein 2** | | | | | | | | | | | | |
| Virus | 328 | 339 | 368 | 627 | 649 | | 17 | 191 | 197 | 295 | | | |
| A/swan/R65/Germany/2006 | L | T | Q | K | I | | | | | | | | |
| A/Thailand/1(KAN-1)/2004 | F | K | R | E | V | | | | | | | | |
| A/chicken/Accra/10/2015 | F | K | R | E | V | | R | D | K | V | | | |
| A/chicken/Ketu/43/2015 | F | K | R | E | V | | C | E | K | M | | | |
| A/chicken/Obuasi/50/2015 | F | K | R | E | V | | R | E | R | M | | | |
| | **Nucleoprotein** | | | | | | | | | | | | |
| Virus | 33 | 270 | 408 | | 398 | | | | | | | | |
| A/swan/R65/Germany/2006 | I | I | I | | | | | | | | | | |
| A/Thailand/1(KAN-1)/2004 | V | V | V | | | | | | | | | | |
| A/chicken/Accra/10/2015 | V | V | V | | Q | | | | | | | | |
| A/chicken/Ketu/43/2015 | V | V | V | | L | | | | | | | | |
| A/chicken/Obuasi/50/2015 | V | V | V | | L | | | | | | | | |

Legend: changes were also observed in the following positions when compared with R65, amino acid positions are referenced to R65: **HA**: 2, 45, 53, 66, 71, 120, 133, 136, 140, 162, 163, 169, 174, 184, 200, 219, 226, 227, 240, 266, 269, 277, 310, 457, 475, 513, 528, 533 **NA**: 8, 20, 29, 46, 50, 58, 75, 99, 143, 172, 238, 250, 264, 269, 316, 319, 346, 366, 369, 398 **PB2**: 64, 195, 255, 292, 340, 389, 390, 394, 451, 483, 495, 511, 660, 676, 677 **NP**: 52, 350, 373, 450, 452

### 4.2.3 Generation of recombinant H5N1 HPAIV isolates from Ghana

To be able to analyse isolated H5N1 HPAIV strains for their zoonotic potential, a series of *in vitro* assays were performed. Viral gene segments from H5N1 viruses isolated from Ghana were first cloned into pHW2000 expression plasmids. Viruses were subsequently rescued using the 8-plasmid system described by Hoffmann and colleagues (Figure 12). Eight plasmids containing viral cDNAs (PB2, PB1, PA, NP, HA, NA, M and NS) were transfected into HEK293T cells. cDNA was inserted between the human polymerase I promoter and a termination sequence and further bordered by RNA polymerase II (pol II) promoter and a polyadenylation site. Because each plasmid contained 2 different promotors, positive-sense viral mRNAs as well as negative-sense vRNAs are synthesisized from the same cDNA template. The molecules from the cellular and viral transcription and translation machineries then interacted to produce infectious IAV. Supernatants from the transfection containing infectious particles were then used to infect MDCK II cells. Presence of CPE signified a successful rescue. An overview of virus strains isolated from Ghana as well as control strains that were cloned, rescued and used in this study are shown in Table 6. We used KAN-1 and R65 viruses as human and avian controls respectively. KAN-1 (A/Thailand/1(KAN-1)/2004 (H5N1)) was isolated from a 6 year old boy in Kanchanaburi, located in the West of Thailand. KAN-1 possesses a multiple basic amino acid cleavage site in HA, lacks the human adaptive signature E627K in PB2 and prefers binding to α2,3 linkage sialic acid cell surface receptors [158]. KAN-1 was therefore used as a mammalian H5N1 control. R65 (A/swan/Germany/R65/2006 (H5N1)) was isolated from a dead whooper swan found in early February 2006 in Ruegen, an island in the south-western part of the Baltic sea in the northern part of Germany. R65 also possesses a multiple basic amino acid cleavage site and the human adaptive signature E627K in PB2. R65 also bore 99% amino acid identity to a H5N1 virus that infected and killed a cat in the same area [159]. R65 was therefore used as avian H5N1 control.

H5N1 recombinant viruses with monobasic HA cleavage sites for all viruses from Ghana as well as control viruses with mono basic HA cleavage sites were also generated. H5N1 HPAIV bearing mono basic cleavage sites were generated so that viruses could be handled in the biosafety level II laboratories.

Table 6. Overview of influenza A virus strains used in this study

| Strain name | Source | PB2 | PB1 | PA | NP | HA | NA | M | NS | Abbreviated as: |
|---|---|---|---|---|---|---|---|---|---|---|
| A/chicken/Accra/08/2015 (H5N1) | chicken | - | - | - | - | - | - | - | - | |
| A/chicken/Accra/09/2015 (H5N1) | chicken | - | - | - | - | - | - | - | - | |
| **A/chicken/Accra/10/2015 (H5N1)** | chicken | √ | √ | √ | √ | √ | √ | √ | √ | **Accra** |
| | | | | | | | | | | |
| A/chicken/Ketu/42/2015 (H5N1) | chicken | - | - | - | - | - | - | - | - | |
| **A/chicken/Ketu/43/2015 (H5N1)** | chicken | √ | √ | √ | √ | √ | √ | √ | √ | **Ketu** |
| A/chicken/Ketu/44/2015 (H5N1) | chicken | - | - | - | - | - | - | - | - | |
| | | | | | | | | | | |
| **A/chicken/Obuasi/50/2015 (H5N1)** | chicken | √ | √ | √ | √ | √ | √ | √ | √ | **Obuasi** |
| A/chicken/Obuasi/51/2015 (H5N1) | chicken | - | - | - | - | - | - | - | - | |
| A/chicken/Obuasi/53/2015 (H5N1) | chicken | - | - | - | - | - | - | - | - | |
| **Control viruses** | | | | | | | | | | |
| A/Thailand/1(KAN-1)/2004 (H5N1) | human | - | - | - | - | - | - | - | - | KAN-1 |
| A/swan/Germany/R65/2006 (H5N1) | swan | - | - | - | - | - | - | - | - | R65 |

Viruses in bold were selected and used for further *in vitro* and *in vivo* analysis. Viral gene segments marked with √ were cloned during this study, - indicates that cloning was not done for these virus strains. All virus strains isolated from Ghana as well as control strains were rescued using the 8-plasmid system previously described (Figure 12).

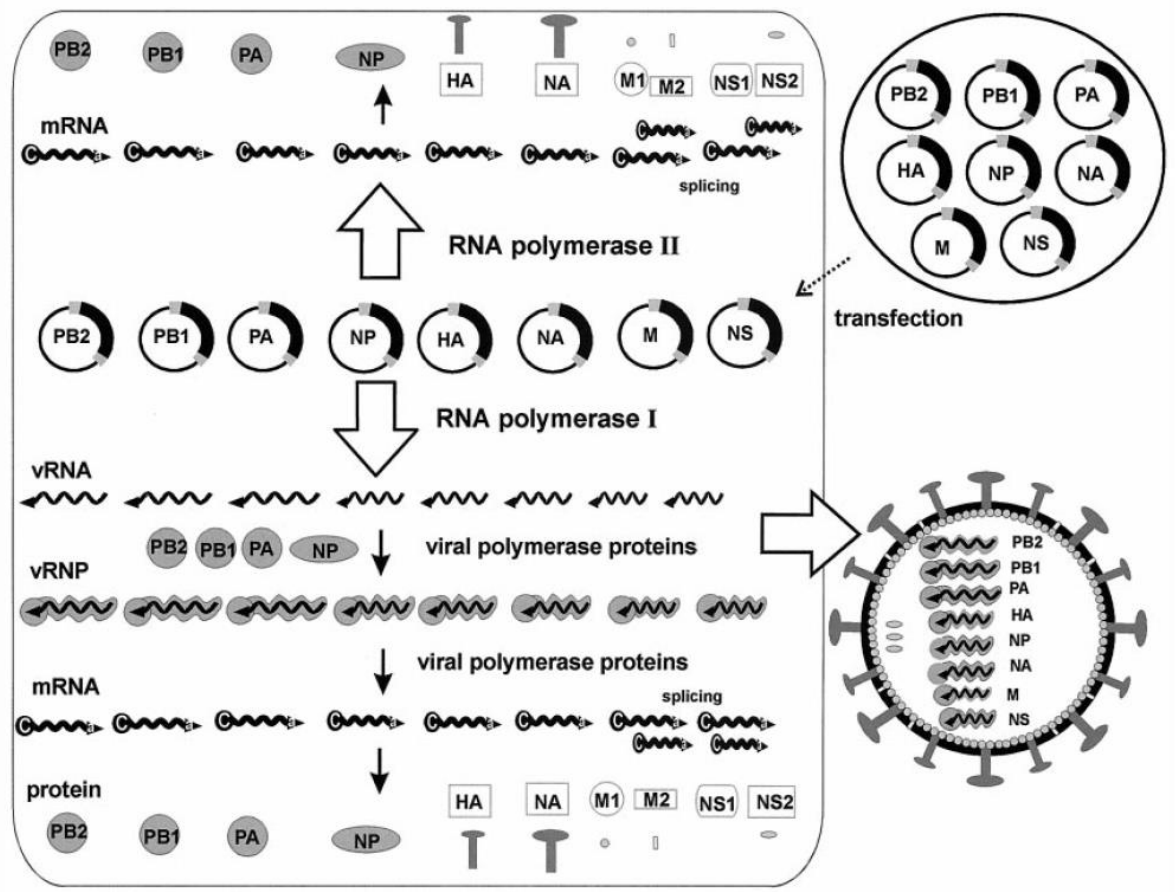


**Figure 12. Overview of influenza virus rescue using the 8-plasmid system.** Viral gene segments are inserted between the RNA polymerase I (pol I) promoter and terminator sequences of pHW2000 expression vectors. This construct is further bordered by an RNA polymerase II (pol II) promoter and a polyadenylation site. The orientation of the two transcription units allows the synthesis of negative-sense vRNA mediated by the viral RNA-Dependent RNA polymerase and positive-sense viral mRNA generated by the cellular polymerase. Therefore, all molecules from the cellular and viral transcription and translation machineries interacted to produce an infectious influenza A virus [138].

### 4.2.4 Thermal stability of H5N1 HPAIV Ghanaian isolates

HPAIV of the subtype H5N1 are still circulating in Ghana and the West African region despite the warm climates and high humidities in these areas. It has been shown that high temperatures and humidity easily inactivate IAV so that they are no longer infectious or transmissible [160-162]. Thermal stability of a virus could give an indication whether the HA would be cleaved at a higher or lower pH [144]. Typically, the HA of human adapted IAV are cleaved at lower pH [9, 38-40]. The lower the pH at which HA is cleaved, the more likely it is the avian virus could infect humans [9, 38-40]. A virus with HA cleaved at low pH is likely to be highly stable in thermal conditions [9, 38-40]. Due to these reasons, the thermal stability of H5N1 HPAIV isolated from Ghana was compared to control viruses KAN-1 and R65. Viruses were subjected to temperatures of 50°C and subsequently infectious virus particles were determined using plaque tests. Thermal stability of H5N1 viruses isolated from Ghana

compared with control viruses is shown in Figure 13. We could observe that at 60 minutes, virus isolate from Obuasi was the least stable virus, with isolate from Accra displaying an intermediate phenotype, presenting greater viable virus than R65, but less than KAN-1. We could observed that, all Ghanaian strains are highly stable at high temperatures, loosing infectivity only after 240 minutes (4 hours), with viruses from Ketu being the most stable, viable after 360 minutes (6 hours). Control viruses KAN-1 and R65 were also viable after 360minutes (6 hours).

Taken together our results showed that H5N1 HPAIV isolates from Ghana exhibit high stability in high temperatures, maintaining infectivity after 4hours (120minutes) at 50°C, with virus isolate from Accra displaying an intermediate phenotype at 60minutes. The least stable virus, from Obuasi, was inactivated after 4hours (120minutes) with viruses from Ketu remaining infectious even after 6hours (360minutes). Control viruses were however more stable than H5N1 HPAIV isolates from Ghana, both remaining viable after 6 hours (360 minutes).

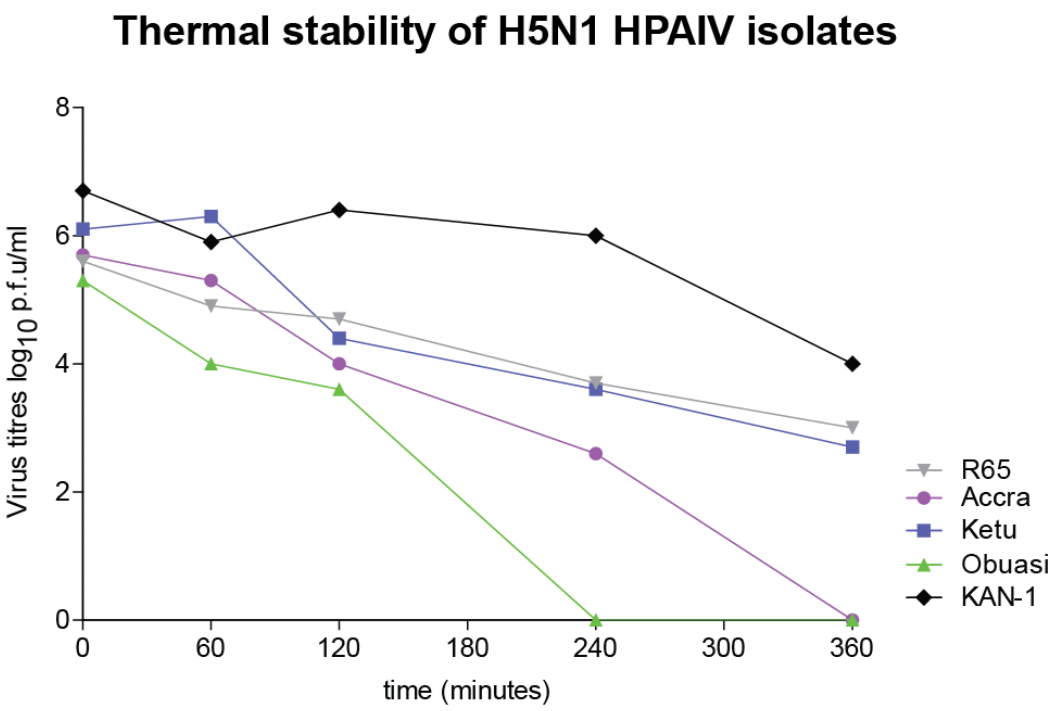


**Figure 13. Thermal stability for H5N1 HPAIV isolates from Ghana in 2015.** Viruses were subjected to temperatures of 50°C for various time points. Virus titres were determined using plaque assays. Graph is a representative of 3 experiments performed and shows virus titres at various time points [144].

### 4.2.5 Receptor specificity of HPAIV H5N1 Ghanaian isolates

For an avian IAV to successfully infect human cells, the virus must be able to switch receptor specificity from SAα2,3Gal to SAα2,6Gal. H5N1 HPAIV isolates from Ghana harboured mutations in HA which had been described to increase virus binding to SAα2,6Gal and enhance transmission in guinea pig models (Table 4). Due to this, the receptor binding specificity for viruses isolated from Ghana as well as control viruses was investigated using HA with modified turkey red blood cells. Included in the assay were two viruses: A/Netherlands/213/2003 (H3N2) and A/Vietnam/1194/2004 (H5N1) which served as internal controls. The H3N2 virus was used as human control virus which was expected to bind to only SAα2,6Gal while the H5N1 virus served as avian control, binding only to SAα2,3Gal. Results showed that all virus strains isolated from Ghana preferentially bound to SAα2,3Gal (Table 7), displaying strong avian tendencies. Control viruses also exhibited strong avian binding to SAα2,3Gal.

We identified mutations: D94N, S133A, S155N and T156A in HA of H5N1 HPAIV Ghanaian isolates, which previously shown to cause increase in virus binding to SAα2,6Gal (Table 4). We could however demonstrate that H5N1 HPAIV isolates from Ghana preferred binding to sialic acids linked to galactose sugars in α2,3 conformation, as opposed to SAα2,6Gal. Both control viruses also preferred SAα2,3Gal.

Table 7. Receptor specificity determination of H5N1 Ghanaian virus isolates.

| | | HA titre (HAU/50µl) | |
|---|---|---|---|
| **Virus** | **Subtype** | **α2,3-linked TRBCs** | **α2,6-linked TRBCs** |
| A/chicken/Accra/10/2015 | H5N1 | 64 | 0 |
| A/chicken/Ketu/43/2015 | H5N1 | 64 | 0 |
| A/chicken/Obuasi/50/2015 | H5N1 | 64 | 0 |
| A/swan/Germany/R65/2006 | H5N1 | 64 | 0 |
| A/Thailand/1(KAN-1)/2004 | H5N1 | 64 | 0 |
| A/Netherlands/213/2003 | H3N2 | 0 | 64 |
| A/Vietnam/1194/2004 | H5N1 | 64 | 0 |

Receptor specificity was determined by a modified Turkey Red Blood Cells (TRBC) haemagglutination (HA) assay. TRBCs were treated with *Vibrio cholerae* NA to remove sialic acids from the cell surface. Optional α2,3- or α2,6-linked SA were resialylated by either α2,3- or α2,6- N-sialyltransferases. The HA assay was then performed with the modified TRBCs. A/Netherlands/213/2003 (H3N2) and A/Vietnam/1194/2004 (H5N1) were used as internal controls for the assay. The table shows average of three independent experiments [145].

### 4.2.6 Neuraminidase activity of H5N1 HPAIV Ghanaian isolates

The NA of IAVs, specifically cleave sialic acids on the surface of the infected cells to facilitate virus release from the cells [6]. This implies that, successful viral infections require active NA. The NA of H5N1 HPAIV isolates from chicken in Ghana harboured various mutations in NA with functions yet unknown (Table 4). Therefore the NA activity of Ghanaian virus isolates compared with control viruses was investigated. For this assay, H5N1 HPAIV bearing mono basic cleavage sites were used in the biosafety level II laboratories. The assay was performed using the fluorescent compound 4-methylumbelliferyl *N*-acetylneuraminic acid (4-MUNANA). The NA cleaves the compound into a 4-methylumbellyferone (4-MU) which fluoresces. Hence, the amount of fluorescence directly correlates with NA activity. The results of this assay showed that H5N1 HPAIV Ghanaian isolates showed less neuraminidase activity compared to control viruses (Figure 14). When compared to neuraminidase activity of R65, Ghanaian virus isolates showed significantly reduced neuraminidase activity ($p<0.001$), with virus isolates from Ketu showing slightly higher neuraminidase activity among all Ghanaian isolates assessed. The neuraminidase activity of the human KAN-1 isolate was highest compared to all other strains.

Taken together, all Ghananian H5N1 HPAIV isolates present an intermediate phenotype between the avian control R65 and the human control KAN-1 strain. These findings may suggest that some adaptive changes have occurred in the NA gene of the Ghanaian isolates that reduce NA activity.

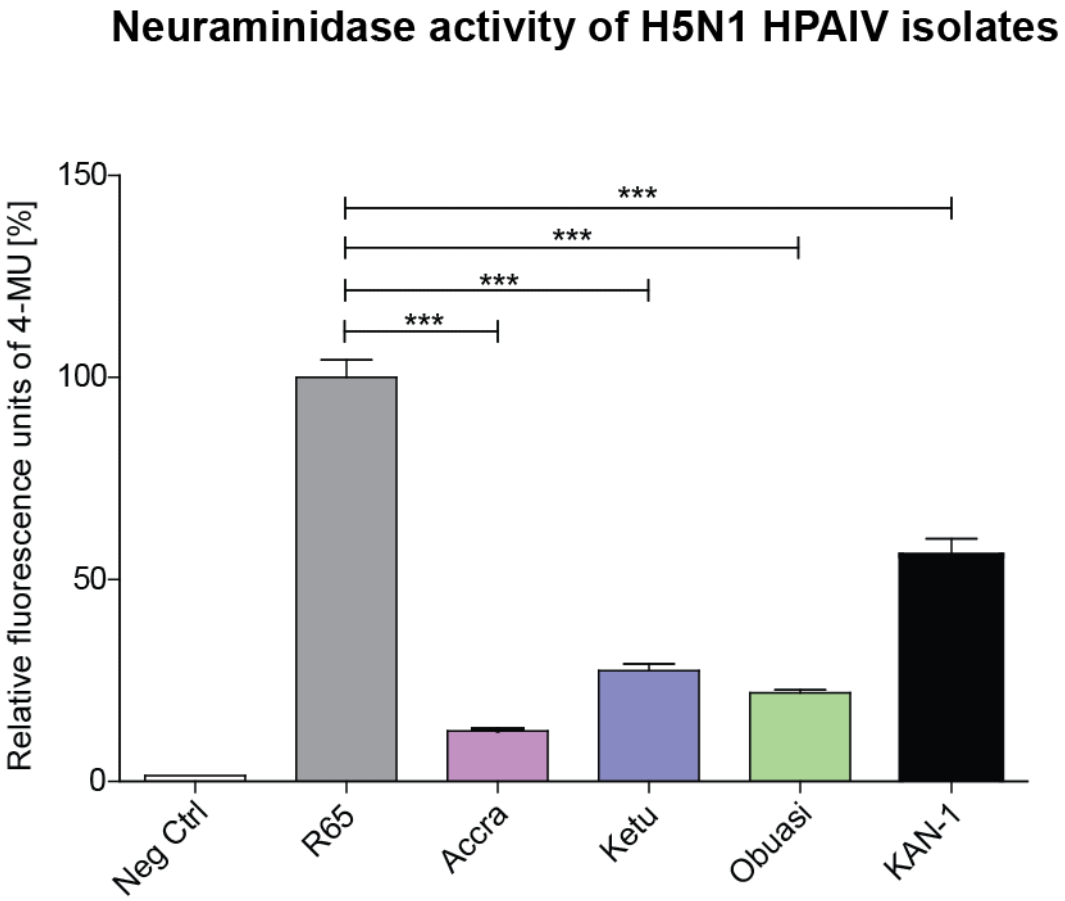


**Figure 14. Viral neuraminidase activity**. Neuraminidase activity was determined using 4-MU-NANA (4-methylumbelliferyl *N*-acetylneuraminic acid) in calcium-TBS buffer (6.8 mM CaCl2, 0.85% NaCl, 0.02 M Tris; pH 7.3) as a substrate. Release of 4-methylumbellyferone (4-MU) fluorescence was determined in a TECAN Safire2 reader at $\lambda_{Exc}$ = 355nm and $\lambda_{Em}$ = 460nm. NA activity was expressed in relative flourescence units of 4-MU. Graph shows the mean and standard error of three independent experiments of triplicates each (n=9). Statistical significance was calculated using unpaired Student's t-test and ANOVA (***p<0.001) [146].

### 4.2.7 Activity of the reconstituted polymerase of H5N1 HPAIV Ghanaian isolates

H5N1 HPAIV isolates from Ghana did not harbour known mutations E627K and D701N in PB2, as well as N319K in NP, all located in the viral polymerase complex, which have been described to enhance polymerase activity of avian viruses in mammalian cells. However, all virus isolates from Ghana harboured a combination of mutations known to compensate for the lack of the mutations described above. These mutations were also shown to rescue viral polymerase activity of avian viruses in mammalian cells when they occurred together (Table 4). Polymerase activity of H5N1 HPAIV Ghanaian isolates was therefore analysed in mammalian cells. We used H1299 (human non-small carcinoma cells derived from lymph nodes) to represent mammalian lung cells. This was done to investigate whether the mutations observed in PB2 for H5N1 HPAIV Ghanaian isolates could rescue the polymerase activity of these avian viruses in the H1299 cells. Polymerase activity was also investigated in avian cells. As Ghanaian strains were of avian origin, we estimated that polymerase

activity will not be restricted in avian cells. Results of the polymerase activity of Ghanaian strains compared with control virus strains are shown in Figure 15. In human cells, polymerase activity of the H5N1 Accra isolate was increased up to 150% compared to the avian R65 control isolate that was set to 100% ($p<0.001$) (Figure 15A). The H5N1 isolates from Ketu and Obuasi showed low polymerase activity similar to the avian R65. The human KAN-1 isolate showed, as expected, highest polymerase activity in human cells. In DF-1 cells, polymerase activity of Ghanaian strains was significantly different, compared to R65, (Figure 15B). The Accra isolate showed slightly higher polymerase activity compared to R65, albeit the Ketu and Obuasi strains showed reduced polymerase activity. Again, KAN-1 presented highest polymerase activity in avian cells.

Taken together, we could show that polymerase activity of the H5N1 Accra HPAIV Ghanaian isolate was highest among all Ghanaian isolates tested compared to the avian R65. However, its activity was reduced compared to KAN-1. This suggests that especially the Accra H5N1 isolate has acquired some adaptive changes in its polymerase that increases polymerase activity in human cells.

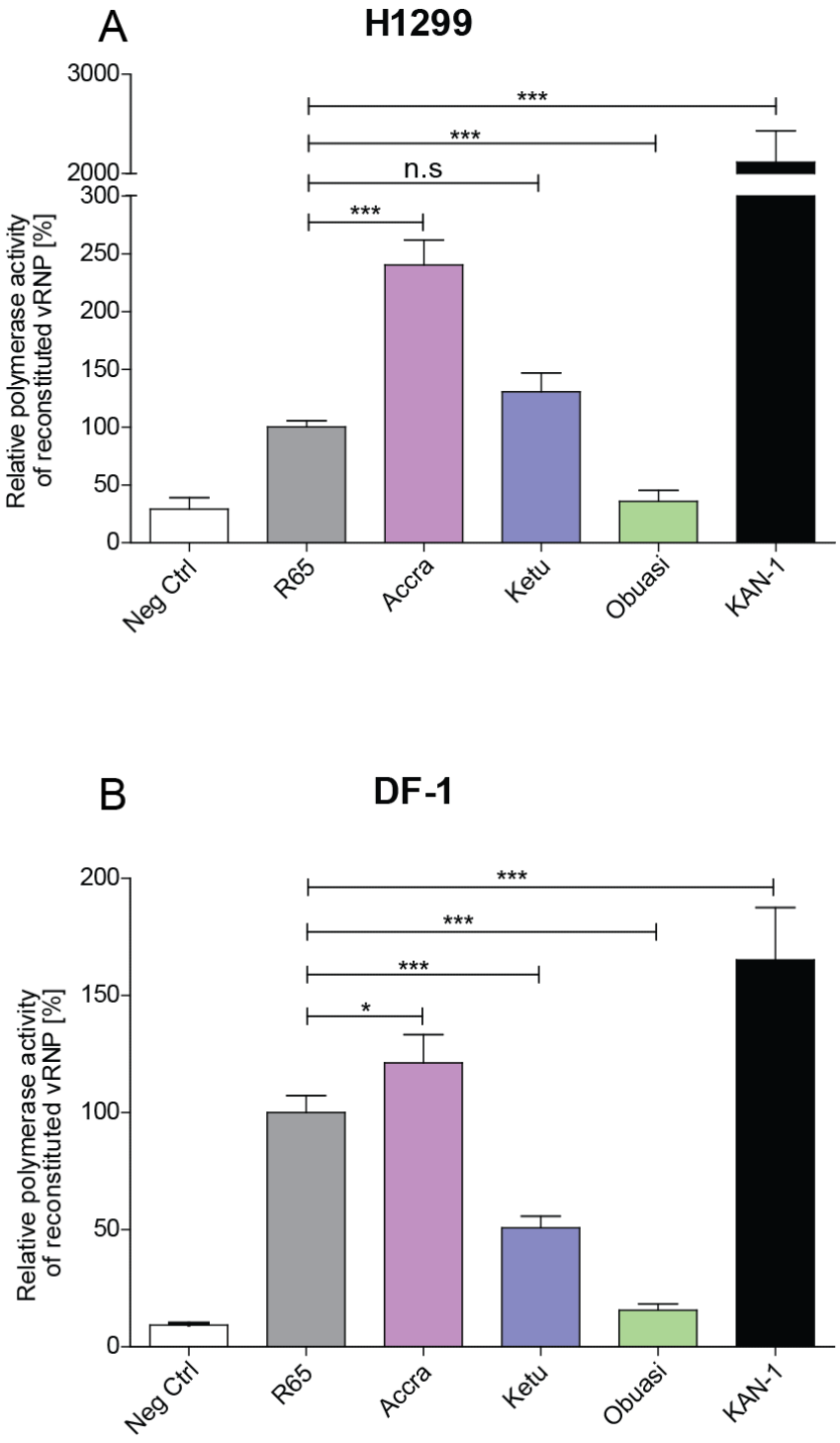


**Figure 15. Activity of the reconstituted vRNP of viruses from Ghana**. Polymerase activity in **A** H1299 and **B** DF-1 cells were determined. Cells were co-transfected with pHW2000 expression plasmids encoding the polymerase genes PB2, PB1, PA and NP of all viruses as well as pPol1-NP-Luc and pRenilla-TK plasmids. Cells were harvested 24hours post transfection (except for H1299cells which were harvested after 48hours) and assayed for luciferase activity. Values were standardized with pRenilla-TK values. Neg ctrl: KAN-1 plasmids without PB2 (pHW2000 empty vector). Graph shows average and standard error of six independent experiments of triplicates each. Statistical significance was calculated using the unpaired Student's t-test and ANOVA (*p<0.05, ***p<0.001) [141].

### 4.2.8 Virus replication kinetics

We investigated whether H5N1 HPAIV isolates from Ghana could fully replicate in mammalian and avian cells. Eventhough polymerase activity was measured as a preliminary indication of repliction competence, it was important to confirm this observation. To investigate the replication competence of H5N1 HPAIV in mammalian and avian cells, we used Calu-3 (cultured human airway epithelial cells) and DF-1 cells.We infected both cells with H5N1 HPAIV at MOI of 0.001. Supernatants were assayed at 24hour intervals and virus titres determined in plaque tests using MDCK cells. Results from the viral replication kinetics are shown in Figure 16. In human Calu-3 cells, Ghanaian H5N1 HPAIV isolates from Accra and Ketu replicated to significantly higher levels compared to the avian R65 but reduced levels than the human KAN-1 (Figure 16A). Only the Obuasi isolate replicated to lowest levels compare to R65 and KAN-1. In avian DF-1 cells, all Ghanaian isolates replicated to lower levels compared to both avian R65 and human KAN-1 (Figure 16B). However, 96h p.i. no differences were detacted among all viruses assessed. In line, the Accra H5N1 isolate that presented high polymerase activity in human cells also presents high replicative fitness in these cells.

These findings suggest that the Ghanaian isolates from Accra and Ketu have acquired some mammalian adaptive signatures that enable them to replicate to elevated titres in human cells.

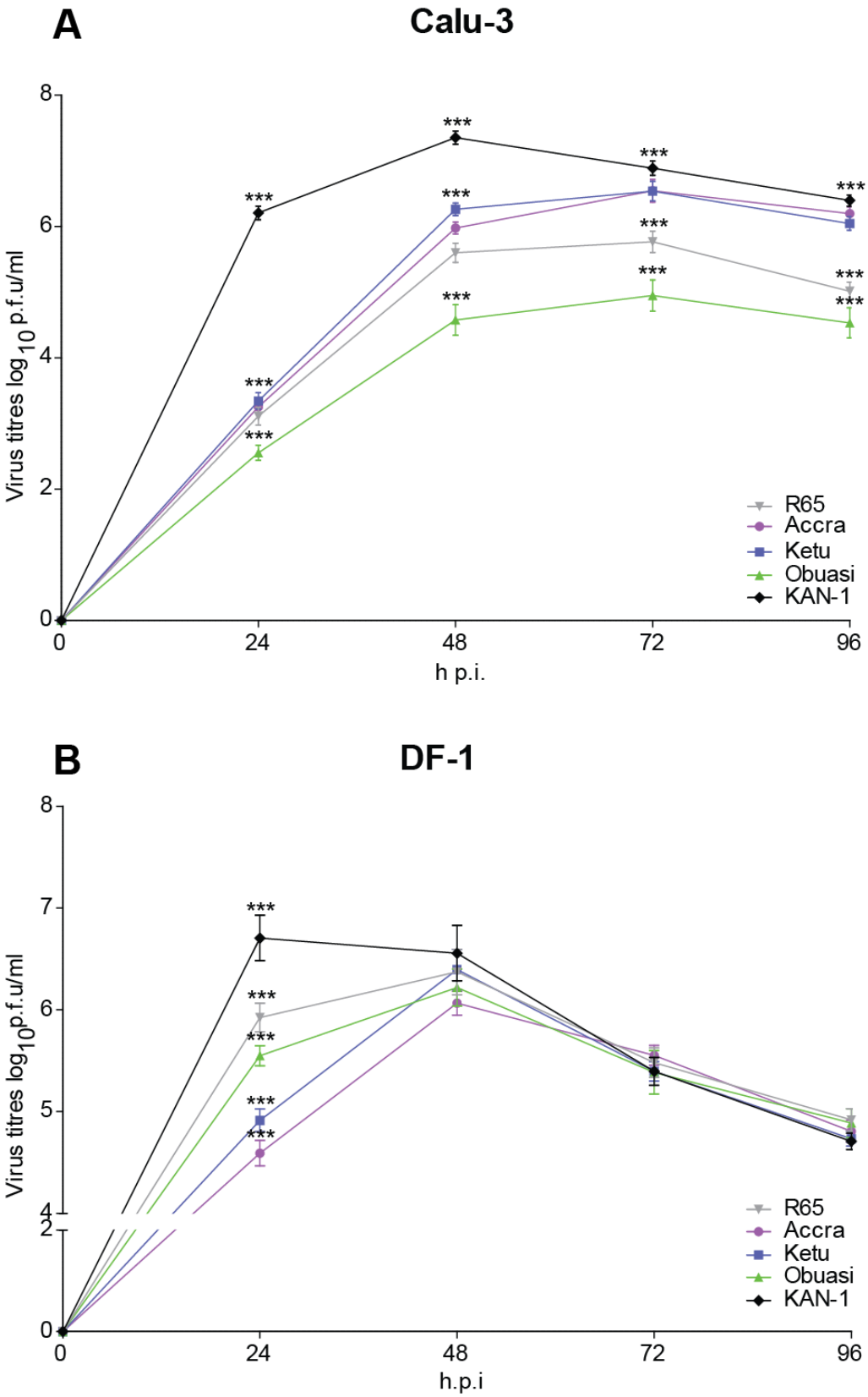


**Figure 16. Classical growth kinetics for H5N1 HPAIV isolates from Ghana in 2015.** H5N1 viruses were used to infect **A** Calu-3 cells and **B** DF-1 cells at MOI of 0.001. Virus titres were determined using plaque tests at various time points (time point 0-96hours). Graph shows average and standard error of three independent experiments of triplicates each. Statistical significance was calculated using the unpaired Student's t-test (***p<0.001) [111].

### 4.3 Disease severity of H5N1 HPAIV isolates from Ghana in the BALB/c mouse model

To determine the pathogenicity and virulence of H5N1 HPAIV isolates from Ghana in 2015, eight weeks old female BALB/cRj (Janvier Laboratories) were infected with $10^2$p.f.u./ml of each virus.

#### 4.3.1 Pathogenicity and virulence of H5N1 HPAIV isolates from Ghana in BALB/c mice

To be able to demonstrate the lethality of H5N1 HPAIV viruses isolated from Ghana *in vivo* in a mammalian model, we infected BALB/c mice with these viruses. We infected mice with $10^2$p.f.u/ml of virus. Control animals received 50µl PBS. All animals were monitored for 14 days for weight loss and survival (Figure 18). All virus strains assessed replicated to similar levels in the murine lungs comparable to R65 and KAN-1, except the Ketu and Obuasi strain at 3d p.i. with reduced titers (Figure 17A). H&E staining of fixed lung slides showed extensive infiltration of alveoli of infected murine lungs (Figure 17B) for all virus isolates. Lung titres correlated with mononuclear inflammation in H&E stainings. In mice, all virus strains caused weight loss (Figure 18A). As expected, KAN-1 showed highest lethality with 100% (Figure 18B). The avian R65 also showed high lethality with approximately 80%. Among the Ghanaian isolates, the Obuasi was the isolate with highest lethality of 100%, while the Ketu and Accra isolates were less lethal. However, these experiments were performed with five animals per group and should be repeated with a larger animal number.

In summary, the Ghanaian isolates all presented pathogenicity with the H5N1 Obuasi isolate presenting highest lethality with 100% comparable to the human KAN-1 isolate.

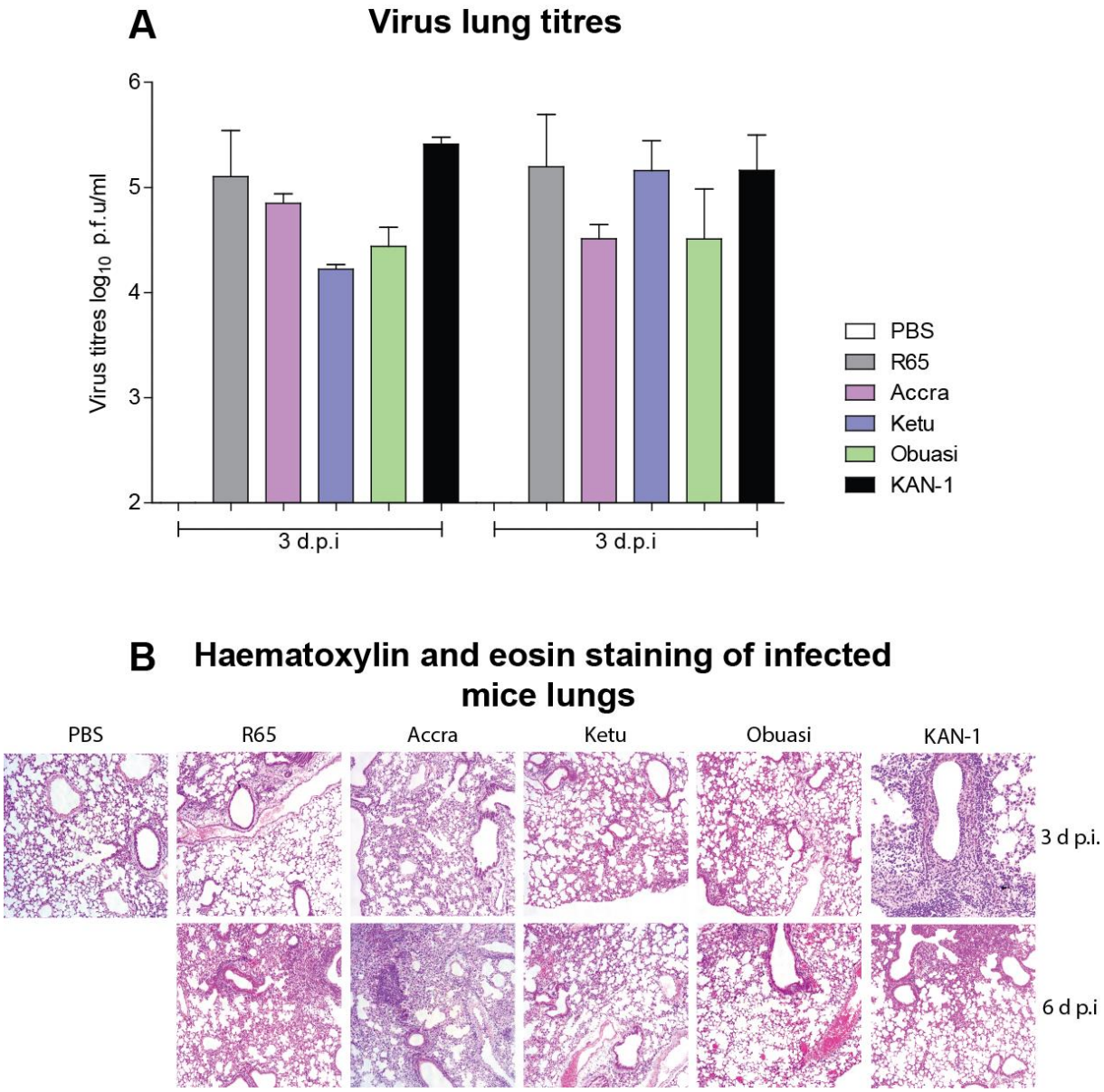


**Figure 17. Pathogenicity of H5N1 HPAIV isolates from Ghana in BALB/c mice**. Eight weeks old female BALB/c mice were infected with $10^2$ p.f.u of each virus (4/5 mice/virus for weight loss and survival and 6 mice/virus for organ harvesting 3 d.p.i and 6d.p.i). Three mice per group were sacrificed 3d.p.i. and 6d.p.i.and lungs were harvested. Virus titres **A** in lungs were determined. Mice lungs were stained with H&E **B**. Pictures show infected mice lungs 3 d.p.i and 6 d.p.i. Lungs show massive infiltration of alveoli [169].

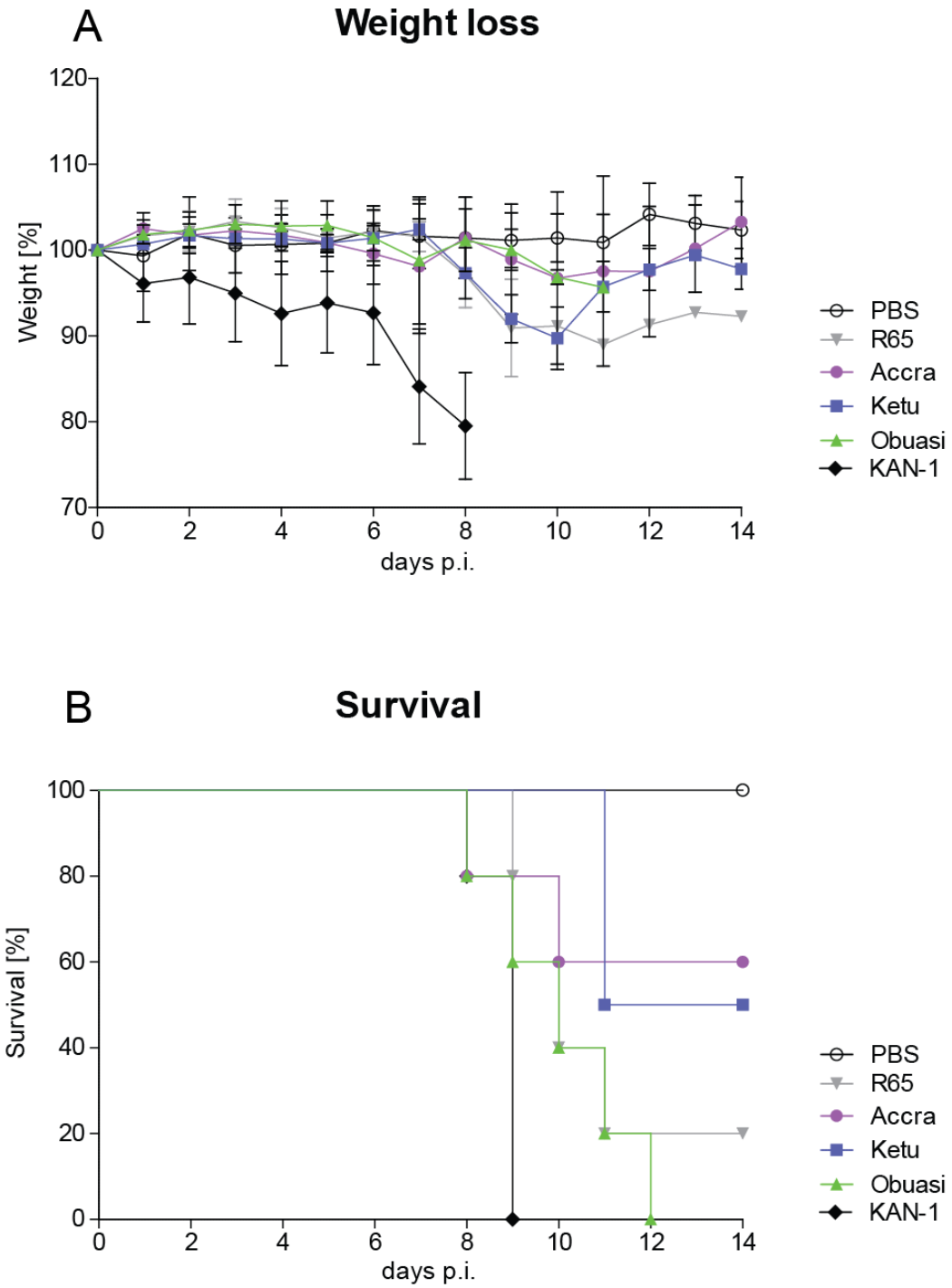


**Figure 18. Lethality of H5N1 HPAIV isolates from Ghana in BALB/c mice**. Eight weeks old female BALB/c mice were infected with $10^2$ p.f.u of each virus (4/5 mice/virus for weight loss and survival and 6 mice/virus for organ harvesting 3d.p.i and 6d.p.i). Mice were monitored for up to 14 days for **A** weight loss and **B** survival. Statistical significance was calculated using the unpaired Student's t-test and ANOVA to assess the differences among the viruses from Ghana [169].

## 5.0 Discussions

In Africa, morbidity and mortality due to IAV infections on the population is considered non significant. This is because the continent is plaqued with several other debilitating diseases. This narrative however changed with the introduction of H5N1 HPAIV among poultry on the continent, leading to huge economic losses and subsequent human infections. Again, confirmation of the 2009 pandemic IAV in many countries on the continent, has highlighted the importance of influenza disease on the continent. In Ghana, the situation is not different from other African countries. Influenza, although diagnosed in the country as far back as 1919 during the 1918 pandemic, was not considered an important disease until the scenarios already discussed. Since then, research on influenza in Ghana has focused on detecting IAVs in the general human population. Other research activities have focused on detecting IAVs among domestic poultry or wild birds. In Ghana however, humans and animals are in constant close contact, therefore the potential for reassortment is an immediate threat. We therefore aimed to bridge this gap by studying IAVs at the human/animal interface. We initially collected and analysed sera from farmers who were in constant contact with animals in a sero-prevalence study, to detect the presence of IAV antibodies. We further identified H5N1 HPAIV, which cuased an outbreak among chicken in Ghana in 2015, leading to the mortality of approximately 1 000 000 chicken. We sequenced these H5N1 HPAIV isolates and performed a rigorous assessment regarding their potential to cross species barriers and transmit from birds to humans.

### 5.1 Influenza sero-prevalence among Ghanaian farmers and animals

In our sero-prevalence study, we tested farmers sera against two different H3N2 IAV strains, a 2009 pandemic IAV strain as well as an H5N1 HPAIV strain. We were able to detect antibodies to HA of influenza A/Aichi/2/1968 (H3N2) present on WSN/33 virus (2.18) to protective levels from all farmers tested (27 farmers). We anticipated this observation because A/Aichi/2/1968 (H3N2) virus had been circulating since 1968 and therefore antibodies were prevalent in various human populations. Against A/Netherlands/213/2003 (H3N2), which is a more recent isolate, up to 50% of Ghanaian farmers exhibited antibodies to protective levels. As this virus was a more contemporary isolate and had not circulated for longer, we expected that less Ghanaian farmers will display protective antibody levels. Antibody titres to the 2009 pandemic IAV A/Sachsen-Anhalt/101/2009 (H1N1) were highest (≥1:40) in older farmers, 40-79 years old, compared to younger farmers. In a study conducted in Germany in 2013 [139], Hackenberg and colleagues observed, that in Germany, generally, high level antibody titres against the 2009 pandemic IAV were prevalent in young adults (18-39 years) when compared with older age groups. Our results were contrary to this observation. Although the sample size for this study was small, it was

interesting to observe that older farmers exhibited high level antibodies to the 2009 pandemic IAV. Pre-existing antibodies to the 2009 pandemic IAV among older groups were previously described. Miller and colleagues could show in 2010, that 23.3% (57/253 samples collected in 2008) of persons aged ≥65years in England possesed pre-existing antibodies to the 2009 pandemic IAV and this could have accounted for the lesser incidence observed among this age group, an indication of a certain level of protection [163]. We speculate therefore that older Ghanaian farmers could possess pre existing antibodies to the 2009 pandemic IAV and this could have accounted for our observation. Farmers sera tested negative to H5N1 HPAIV viruses, dislaying no antibodies to R65. Samples were collected before the outbreak of H5N1 HPAIV among poultry in Ghana, therefore this observation could demonstrate that H5N1 HPAIV viruses were most likely not circulating in Ghana before the outbreak. Just like many African countries, Ghana does not vaccinate against influenza. Data such as this, demonstrating antibodies to IAV for Ghanaian farmers is important in informing policy change and the importance of continuous surveillance among humans and animals.

Risk factors for acquiring IAV infection from animals as well as transmission to animals were assessed. In this this study, we observed that all farmers never washed their hands before handling animals, all farmers never wore facemasks, 96% never wore aprons, 80% never wore gloves while handling animals, 63% always wore boots and all farmers washed their hands after handling the animals. Although the main mode of human to human transmission of influenza is by large virus-containing droplets (>5µm) [6], contact transmission is also a major way to spread the infection [160-162]. Lowen and colleagues could show using a guinea pig model that high temperatures (30°C) block aerosol transmission but not contact transmission. Based on this, they propose that the main mode of transmission in the tropics could be via contact transmission [164]. Assessment of the risk factors for transmission among Ghanaian farmers and average temperatures in Ghana (24-30°C) suggest that, the potential for transmission of infection to and by animals through direct contact is high. Basic infection control methods such as hand hygiene has proven to be an effective way of reducing transmission [160-162]. However, farmers did not wash their hands prior to handling animals. An indication that, animals could be easily infected by sick farmers. We could demonstrate for the first time with this study, presence of antibodies to different IAVs among farmers in Ghana. This finding indicates that Ghanaian farmers were exposed to IAVs. It is important therefore that education among Ghanaian farmers should be intensified. Ghanaian farmers should understand the importance of protecting themselves and the animals they work with, to curb potential reassortant events. In the wake of the presence of different subtypes of avian IAVs circulating in Africa, education is urgent.

We did not detect the presence of antibodies in animal sera (poultry and pigs) to HA of influenza A/Aichi/2/1968 (H3N2) containing the internal genes of A/WSN/33 virus (2.18), A/Netherlands/213/2003 (H3N2), A/Sachsen-Anhalt/101/2009 (H1N1) as well as A/swan/Germany/R65/2006 (H5N1) viruses. No antibodies to any of the viruses were detected for animal sera. The fact that in this this study, we failed to detect antibodies to IAVs in animals does not suggest that these animals are not exposed. Adeola and colleagues in 2016 could show the presence of human influenza H3N2 viruses among 4% (2/50) of pigs tested in the Ashanti region of Ghana [97]. Again, Meseko and colleagues demonstrated recently (April, 2018), that 8.4% (42/500 samples tested) of pig sera contained antibodies to H5N1 viruses of the clade 2.3.2.1c in pigs in Nigeria, an indication that the pigs had been exposed to the virus. This observation, they attributed to increased circulation of H5N1 HPAIV among poultry in Nigeria which has led to a total breakdown of control measures [165].

Currently in Ghana, H5N1 HPAIV as well as H9N2 viruses are co circulating among poultry [93]. We cannot predict when control measures in Ghana could also break down, leading to spill over events into other animals such as pigs. It is therefore recommended that surveillance activities be intensified, so that IAV occurences in the animal population can also be monitored.

### 5.2 Phylogenetic and genetic characterization of H5N1 HPAIV isolates from Ghana

In April 2015, an outbreak of H5N1 HPAIV among domestic poultry was recorded, leading to the death and culling of ~100,000 chicken in Ghana. The outbreak spread very rapidly within a short time among poultry in the country. Prior to this outbreak in Ghana, Nigeria had started reporting outbreaks of H5N1 HPAIV among poultry from January 2015. We obtained samples from dead chicken from this outbreak, isolated the viruses and analysed sequences for the major viral genes involved in viral pathogenicity and adaptation: HA, NA, PB2 and NP. We further compared our sequences with the Nigerian outbreak strain. Genetic and phylogenetic analysis of viral genes sequenced concluded that, isolates from Ghana displayed >90% amino acid homology with the outbreak strain from Nigeria. All viruses from Ghana grouped very closely into the same clade 2.3.2.1c, with the outbreak strain from Nigeria, confirming a close relationship with the Nigerian strain. Ghanaian as well as Nigerian isolates also clustered very closely with H5N1 HPAIV isolated from a fatal human case in Alberta Canada. We further observed that all viruses from Ghana harboured substitutions in HA (D94N, S133A, S155N, T156A), which had been associated with increased virus binding to human-type α2-6-linked sialic acids [92]. Reports of Oseltamivir (NA inhibitor) resistance among patients treated for HPAIV H5N1 [166] and also some H5N1 HPAIV virus strains

[167] have been recorded. These observations are disturbing because most countries have stock piles of Oseltamivir in case of a pandemic caused by H5N1 HPAIV [168]. Ghanaian virus strains however, did not harbor drug resistance mutations. Nevertheless, since these H5N1 HPAIV are still circulating in the country as well as in other West African countries, continuous surveillance is required to enable early detection of drug resistance strains. Amino acid motifs E627K or D701N (never observed in combination) present in PB2 and known to increase replication and virulence in human hosts were not observed in Ghanaian isolates [92]. These mutations are known to enhance binding to importin-α isoforms, leading to increased import of vRNPs into the nucleus and also enhance polymerase activity [111]. A group of compensatory mutations were however observed in PB2 which were previously described to increase pathogenicity in mice. Nucleoprotein sequences of the Ghanaian 2015 outbreak strain showed 99% homology with the Nigerian 2015 outbreak strain, further confirming how closely related the viruses were. We concluded from our observations that, H5N1 HPAIV isolated from chicken in Ghana, were most likely imported into the country from Nigeria. Migratory bird movements and human activities were implicated in the introduction of the virus to the African continent. Because intercountry borders in West Africa are porous due to frequent trading activities and human movement, spread of this virus through the sub-region was uninhibited. In Ghana, spread was attributed to movement of poultry in-country. Mobile dealers in chicken moved their goods (sometimes carrying them on their heads) and sold them from house to house. Again, live bird markets were thought to have aided in the rapid spread of the virus throughout the country. Because H5N1 HPAIV of clade 2.3.2.1c had previously caused deaths in humans, the potential risk for transmission from infected poultry to humans was a major concern [92]. We therefore further analysed H5N1 HPAIV isolates from chicken in Ghana for their ability to transmit and infect humans.

### 5.3 *In vitro* characterization of H5N1 HPAIV isolates from Ghana in 2015

We analysed H5N1 HPAIV isolates from Ghana in 2015 for their zoonotic potential. Frequently, viruses from H5N1 HPAIV outbreaks in West Africa are sequenced and characterized, based on sequence data. In this study, we further analysed viruses for their potential to cause zoonosis. Again, Ghanaian isolates belonged to a clade of virus from which fatal human cases had been recorded. It was therefore imperative for the country to be completely aware of the potential of these virus strains to cause zoonosis. We anticipate that our observations could contribute to a policy change on the use of influenza vaccines in Ghana.

To investigate zoonotic potential of our H5N1 HPAIV Ghanaian isolates extensively, we first cloned viral genes into pHW2000 expression vectors. We subsequently generated

recombinant viruses via virus rescues, which we used in *in vitro* assays. Two H5N1 HPAIV strains previously described (4.2.3) were included as controls for all assays. Briefly, the study used KAN-1 and R65 viruses as human and avian controls, respectively. KAN-1 (A/Thailand/1(KAN-19/2004 (H5N1)) isolated from a 6 year old boy in the West of Thailand, possesses a multiple basic amino acid cleavage site in HA, lacks the human adaptive signature E627K in PB2 and prefers binding to α2,3 linkage sialic acid cell surface receptors [158]. R65 (A/swan/Germany/R65/2006 (H5N1)) isolated from a dead whooper swan found in early February 2006 in Ruegen in the northern part of Germany, possesses a multipla basic amino acid cleavage site and the human adaptive signature E627K in PB2 [159].

### 5.3.1 H5N1 HPAIV Ghanaian isolates are stable at high temperatures

The IAV HA protein is responsible for attachment of the virus to cell surface receptors of susceptible host cells. The protein is made up of 2 distinct sub units (HA1 and HA2) linked together by a disulfide bond. After an IAV enters the host cell, HA undergoes an irreversible conformational change, leading to the fusion of the endosomal membrane with the viral membrane and the eventual release of vRNPs into the cytoplasm [6, 17, 99, 100]. This conformational change is triggered when the pH inside the endosome reaches a certain threshold. H5N1 HPAIV HA trigger membrane fusion at pH 5.5 to 6.0, while human adapted IAVs trigger membrane fusion at pH 5.0 to 5.5 [38-40]. It was previously shown that HA heat stability as well as pH-dependent membrane fusion, contributed to droplet transmission of H5N1 HPAIV transmission in mammals [103, 104, 169, 170]. When IAVs undergo heat treatment at 50°C and neutral pH, a conformational change is induced which mimics that which is triggered by low-pH inside the endosome of infected cells [171]. Hanson and colleagues could show that H5N1 HPAIV HA mutatant viruses harbouring mutations that conferred heat stability, could trigger membrane fusion of HA at a more acidic pH [144]. Thermal stability and reduced pH threshold for membrane fusion were critical for transmissibility in ferrets [144]. This implies that, the more stable a virus is, the more likely it is to trigger membrane fusion at much acidic pH, which is critical for transmission. Hanson and colleagues selected their heat stable viruses after 2 hours of heat treatment [144]. In our study, we subjected H5N1 HPAIV isolates to heat treatment at 50°C for 6 hours. All viruses from Ghana were viable after 120 minutes (2 hours), with viruses from Ketu showing the most heat stability, with viable virus present after 360 minutes (6 hours). We could also observe that at 60 minutes, virus isolate from Obuasi was the least stable virus, with isolate from Accra displaying an intermediate phenotype, with more viable virus present than R65, but less than KAN-1. Control viruses KAN-1 and R65 were also stable after 6 hours. Such highly stable viruses could trigger membrane fusion of HA at a more acidic pH, making it possible for such viruses to be able to transmit among mammals. Another implication is that,

such viruses are then not easily degraded in the environment and are available for onward transmission. Perhaps this could explain why these H5N1 HPAIV are still present in the West African sub-region with on going transmission among poultry.

In summary, we observed that H5N1 HPAIV isolates from Ghana are highly stable at high temperatures of 50°C. This indicates that these viruses are not easily degraded and are easily further transmitted. Another implication is that, such viruses could trigger HA fusion at lower pH, a characteristic of human adapted IAVs. Virus isolate from Accra, at 60minutes display an intermediate phenotype, presenting virus titres between R65 and KAN-1.

### 5.3.2 H5N1 HPAIV isolates from Ghana prefer binding to SAα2,3Gal sialic acids

The HA protein as a host restrictive protein has been discussed in section 1.5.1. Human adapted IAVs preferentially bind N-acetylneuraminic acid attached to the penultimate galactose sugar by an α2,6 linkage (SAα2,6Gal), while avian viruses prefer the α2,3 linkage (SAα2,3Gal). For an avian virus to be able to infect a mammalian cell, replicate and efficiently transmit the infection, changes in the viral genome should lead to a switch in receptor recognition [15, 17]. This could be crucial for the generation of a virus with pandemic potential. Through genetic analysis we could observe that H5N1 HPAIV isolates from Ghana harboured mutations which were described to enhance virus binding to human SAα2,6Gal (Table 4) [92]. We therefore determined receptor specificity for H5N1 HPAIV isolates from Ghana. We observed that all viruses from Ghana preferentially bound to SAα2,3Gal modified TRBCs, an indication that the viruses were very avian in their recognition and binding to cell surface receptors. Although mutations in HA that had been described to increase virus binding to human-type α2-6-linked sialic acids were observed for these viruses, these mutations seemed not to have any effect on the binding preference of these viruses. We further observed that KAN-1 and R65, our control viruses, also preferentially bound to SAα2,3Gal. KAN-1 as a human isolate, still maiantained a lot of avian properties such as avian receptor specificity [158]. However, as has been demonstrated by KAN-1 and previously observed elsewhere [172], avian viruses can infect humans directly, without reassortment or receptor switch.

Taken together, we could show that mutations, which were previously described to increase virus attachment to human SAα2,6Gal, did not have an effect on the viral phenotype. Consequently H5N1 HAPIV isolates from Ghana exhibited avian properties in their receptor recognition.

### 5.3.3 H5N1 HPAIV isolates from Ghana exhibit reduced neuraminidase activity

The NA of influenza viruses cleaves sialic acids from the surface of infected cells, facilitating release of newly formed progeny virus particles [22, 23] which are then available to infect neighbouring cells. Due to this function of NA, HA and NA of influenza viruses need to be in perfect balance for a successful infection [6]. It has been shown that NA activity directly correlates with transmission. Subsequently, increased NA activity leads to increased virus transmission [173, 174]. Studies have demonstrated that reduced NA activity IAV viruses, lead to reduced transmission in guinea pig and ferret models [173, 174]. However, a review of NA resistance mutations on NA activity and transmission in animal models concluded that, because H5N1 HPAIV are able to replicate so efficiently, limited NA activity does not affect transmission of these viruses [175]. In this study, we observed several mutations in NA which have yet to be published (Table 4)[92]. We therefore investigated the effect of these mutations on NA activity. We analysed NA activity of H5N1 HPAIV from Ghana using a fluorescent substrate: 4-MUNANA compared with our control viruses KAN-1 and R65. Compared to our control viruses, all Ghanaian virus isolates showed significantly reduced NA activity (ranging from 10% to 30%), displaying intermediate phenotypes, between R65 and KAN-1. Virus isolates from Ketu show a slightly higher NA activity than other isolates from Ghana. Our observations indicate that NA of Ghanaian isolates is not as active as that of control viruses. However, the H5N1 HPAIV are still circulating in Ghana and other West African countries, confirming the observation that HPAIV H5N1 replicate so efficiently that limited NA activity does not affect transmission.

In summary, we observed that NA activity of Ghanaian strains compared with control viruses KAN-1 and R65 is reduced, with Ghanaian isolates displaying an intermediate phenotype. Our findings suggest that mutations observed in NA could have a negative effect on NA activity, accounting for the considerably reduced NA activity.

### 5.3.4 H5N1 HPAIV isolates from Ghana show increased polymerase activity in mammalian cells

The polymerase complex of IAVs is responsible for transcribing and replication of the viral genome inside the nucleus of an infected cell. The viral polymerase complex interacts efficiently with host factors for a successful infection [6]. Mutations present in the polymerase complex of avian viruses have been shown to enhance replication in part by improved import of vRNPs into the nucleus as well as enhanced interaction of the polymerase complex with host factors, leading to adaptation of HPAIV to the new host [110, 111]. The viral polymerase may therefore be a driving force behind the early evolution of IAVs in a new host, resulting in new pandemic viruses [110, 111]. *In vitro*, we measured polymerase activity with a vRNP

reconstitution assay (3.6.1). We measured polymerase activity in H1299 cells (human non-small carcinoma) and DF-1 cells (chicken fibroblasts). We observed in H1299 human lung cells, when compared with R65, that H5N1 HPAIV Ghanaian isolates show increased polymerase activity. Virus isolate from Accra especially showed 150% increment in polymerase activity compared to R65. Ghanaian isolates from Ketu show a slight increment while Obuasi show restricted polymerase activity. KAN-1 displayed highest polymerase activity in H1299 cells, as expected. Avian polymerase activity in mammalian cells can be rescued by adaptive mutations in PB2 (E627K or D701N) as well as in NP (D701N) [110, 111]. H5N1 HPAIV isolates from Ghana did not harbour these mutations but rather a group of compensatory mutations which had been shown to enhance polymerase activity and increase virulence in mice [92, 149]. Our observations confirm that these adaptive mutations may have rescued polymerase activity of H5N1 HPAIV from Accra and to a large extent Ketu. H5N1 HPAIV isolate from Obuasi showed restricted polymerase activity in mammalian cells. This observation, confirmed that the avian polymerase complex functions poorly in the mammalian host [111]. Adaptive mutations observed in PB2 of the Obuasi isolate did not seem to affect the viral phenotype as they could not rescue polymerase activity of this isolate in mammalian cells. As expected polymerase activity of KAN-1 was highest in both avian and mammalian cells. Our observations confirm that KAN-1 was fully adapted to the mammalian host. Polymerase activity of H5N1 HPAIV isolates in DF-1 cells compared with R65 was significantly different from each other, with isolate from Accra again showing the highest polymerase activity. A genetic comparison among our Ghanaian H5N1 HPAIV isolates (Table 5) revealed differences among the virus isolates. These amino acid changes, could account for the differential polymerase activities observed. We however require further studies to confirm this observation.

Taken together, we could show that Ghanaian H5N1 HPAIV isolates show an increased polymerase activity in a mammalian cell line, with virus isolates from Accra showing highest polymerase activity. This observation could indicate that mutations observed in PB2 could have compensated for the lack of E627K or D710N mutation and rescued polymerase activity of this virus isolate in mammalian cells.

### 5.3.5 H5N1 HPAIV isolates from Ghana replicate effectively in human lung cells

Polymerase activity determination is an indirect indicator of replication competence of a virus in a given cell. Because of this, we confirmed replication competence of Ghanaian virus strains in human lung epithelial cells and chicken cells. We observed that KAN-1 showed peak viral growth at 48 hours in Calu-3 cells (human lung epithelial cells), as expected. H5N1 HPAIV isolates from Accra and Ketu also demonstrated appreciable growth in Calu-3 cells,

growing to titres of ~$10^6$ p.f.u/ml at 48hours. Replication of virus isolates from Obuasi was restricted in Calu-3 cells. These data directly correlates with our observations for polymerase activity measurements. Our observations indicate that H5N1 HPAIV Ghanian isolates from chicken in Ghana displayed replication competence in mammalian cells, in line with observations from polymerase activity. In DF-1 cells, at 24 hours, viruses replicated to significantly different titres, with KAN-1 achieving highest titres. However, after 24 hours post infection, there was no significant difference in replication among the viruses. This data, showed that H5N1 HPAIV Ghanaian isolates replicated efficiently in mammalian and chicken cells.

Taken together, we could show that H5N1 HPAIV isolates from Ghana replicated efficiently in mammalian cells and chicken cells. Our observations suggest that mutations in H5N1 HPAIV Ghanian isolates may have mediated adaptation to mammalian cells.

### 5.5 *In vivo* characterization of H5N1 HPAIV isolates from Ghana 2015

To determine the virulence and pathogenesis of HPAIV isolated in chicken in Ghana in 2015 in mice, eight week old female BALB/c mice were infected. Mice have been used as convenient small mammalian animal models in influenza research over the years. This is mainly because they are easy to maintain, low-cost, with well-charatetrized genetics. Another advantage is that there is a wide range of tests available for mice, this makes it easier to test mouse samples [148, 176]. Mice are however not the natural hosts of influenza virus infections, therefore human origin viruses mostly H3N2 viruses will have to be adapted to mice [176, 177]. Again, mice when infected with influenza viruses, do not shed viruses through the respiratory tract, unlike the situation in humans. Despite these challenges, the mouse model is a good system to check for the virulence of an IAV. In BALB/c mice for eg., illness can be detected by such measurable physical signs in the animal such as lethargy, weight loss, ruffled fur and huddling. These can then be used as a lethality indicator: mouse lethal dose ($MLD_{50}$) [147, 176, 177]. Again, mice produce cytokines in the lungs which can be measured inclusive IFN-α, TNF-α, and IL-6 which can be detected in mouse lung homogenates. It is believed that cytokines contribute largely to recruitmenat and activation of mouse specific immune cells. Histology analysis of infected mice lungs show mainly monocyte/macrophages in the lungs. The extent of infiltration then correlates with the virulence of the virus [176-178]. In recent times, it has been observed that, H5N1 HPAIV viruses, replicate efficiently to high titres in the lungs of mice without prior adaptation [179-181]. Two commonly used in-bred strains in influenza research are BALB/c and C57BL/6J mice. The main difference between BALB/c and and C57BL/6J mice is that BALB/c mice are genetically engineered to mount a Th2 humoral immune response (cytokines: IL-4, IL-6 and

IL-10) while C57BL/6J mount Th1 cellular immune response (cytokines: TNF-α, MCP-1). For this study, we used BALB/c because it was previously shown that H5N1 infections was more virulent in BALB/c mice than C57BL/6J mice [148].

### 5.5.1 Virulence of H5N1 HPAIV isolates from Ghana in BALB/c mice

Female BALB/c mice were infected with $10^2$ p.f.u/ml HAPIV and monitored for 14 days for weight loss and survival. All mice infected with KAN-1 died, 9 days post infection, displaying 100% lethality. Our observation confirmed a previous study where all mice infected with KAN-1 died 9 days post infection [53]. These observations also correlated with our *in vitro* data where KAN-1 showed highest reactions for all analyses. R65 infected mice showed slightly reduced lethality (60% lethality). H5N1 HPAIV isolates from Ghana showed varied lethality. Mice infected with isolates from Obuasi showed highest lethality of 100%, when all mice died 12 days post infection, with infected mice from Accra showing the least lethality and viruses from Ketu showed intermediate phenotype, with 50% lethality. Our observations with vruses from Obuasi *in vivo* did not correlate with our *in vitro data*. Several reasons could account for this. Previous studies have observed that increased polymerase activity correlates with increased virulence in mice[111]. However, Otte and colleagues observed in 2015 that, reduced polymerase activity correlated with increased virulence in mice for 2009 H1N1 pandemic viruses [145]. It is possible, that what Otte and colleagues observed for H1N1 pandemic viruses, could be the case for some H5N1 HPAIV. Another reason could be that the number of mice we used in these experimenst (5 mice per group) were not enough to observe significant differences and therefore the experiment needs to be repeated with larger mouse numbers. A third reason could be that mammalian adaptation may be mediated by other viral genes besides the viral polymerase, such as NS1. It has been previously shown for the 1918 pandemic virus [182], that NS1 was efficiently adapted to human cells, hijacking the interferon pathway and leading to effective viral replication. Therefore it is possible, that adaptation may be mediated by other viral genes. Furthermore, the entry point for H5N1 HPAIV into Ghana was in Accra. The infection then spread through the country, to Ketu and Obuasi. It is also entirely possible, that as the virus travelled through the country it may have acquired further mutations that may have led to its increased virulence in mice, an indication that the virus may be adapting to causing infections in mammals (Table 5). All H5N1 HPAIV isolates from Ghana showed pathogenicity and virulence in BALB/c mice. It has been shown that, virulence of H5N1 HPAIV in humans and BALB/c mice correlates with elevated pro inflammatory cytokine response [148]. Although this study did not measure cytokines, it is possible that high virulence and pathology observed in BALB/c mice could in part be due to other factors, besides high replication competence, such as high cytokine responses. Three days and 6 days post infection, mice were sacrificed and lungs removed to

check for virus titres using plaque tests in MDCK cells. At 3 days post infection, all viruses replicated to titres as high as ~$10^5$ p.f.u/ml. These observations correlated with weight loss and lung pathology, where massive infiltration of alveoli was observed. As expected, virus titres in the lungs of KAN-1 infected mice were the highest (~$10^6$ p.f.u/ml) and correlated with lung pathology, weight loss and survival. These observations were also made with studies involving KAN-1 mouse infections where at 3 days post infection, lung titres were equally high [145]. There were no significant differences among viral replication in the lungs of mice infected with viruses from Ghana. Eventhough viruses from Accra replicated to the highest among Ghanaian virus strains at 3 days post infection, this did not translate to huge weight loss as most mice infected with viruses from Accra did not loose much weight compared to viruses from Ketu and Obuasi. Again survival rate was the highest among the viruses from Accra (3/5 animals survived). However, high replication in the lungs correlated with massive lung infiltration for viruses from Accra. It is possible that host factors could account for this high survival rate of the mice infected with viruses from Accra. Lungs of mice infected with viruses from Obuasi showed appreciable lung titres (~$10^{4.5}$ p.f.u/ml) at 3 days post infection and hardly changed at all at 6 days post infection. These observations translated into high lung pathology and virulence. In contrast viral lung titres increased at 6 days post infection compared with 3 days post infection. This observation has also been shown by other researchers, where lung titres of mice infected with H5N1 HPAIV increase 6 days post infection [183]. Massive infiltration of alveoli with monocytes/macrophages led to severe lung damage and eventual death of the mice. This observation confirms what has been shown by other reports where infection with HPAIV leads to extensive lung damage [145, 183]. Our study confirms that the levels of virus lung titres correlates with the amount of virulence observed for infected mice [164]. We conclude that mutations harboured by H5N1 HPAIV isolated from Ghana in 2015, could mediate the adaptation of these avian viruses to the mammalian BALB/c mouse host.

Taken together, we could show that mutations observed in H5N1 HPAIV isolates from Ghana mediated adaptation of these virus isolates to the murine host. H5N1 HPAIV Ghanaian isolates therefore exhibit zoonotic potential. This finding is of great significance for Ghana. Based on these, education among the general Ghanaian population should be intensified. Urgent steps are needed to curb the spread of these H5N1 HPAIV throughout the country. Our findings confirm that host adaptation of avian viruses to the mammalian host is a complex process mediated by a wide range of viral and host factors.

## 6.0 Conclusion

In Ghana, morbidity and mortality due to IAV infections are considered as negligible. However, outbreaks of H5N1 among poultry and records of the 2009 pandemic IAV have changed this observation. Current studies on IAV in Ghana either concentrates on the presence of the virus in the human population or in the animal population. Since IAVs are easily transmitted from animals to humans, consequently causing severe disease and death, this study sought to study IAVs in Ghana on the human/animal interface.

To do this, we tested sera from Ghanaian farmers as well as poultry and pigs for the presence of antibodies to three different strains of IAVs as well as a H5N1 HPAIV strain. We also analysed risk factors for IAV transmission for Ghanaian farmers. We thoroughly analysed H5N1 HPAIV isolates from an outbreak among chicken in 2015 *in vitro* and *in vivo* for their zoonotic potential. Subsequently, we could show the presence of antibodies to all three IAV strains from the sera of Ghanaian farmers. We detected no antibodies to the H5N1 HPAIV strain. Sera from animals did not contain antibodies to any of the IAVs we tested for. Risk factor assessment indicated that, Ghanaian farmers were at high risk for zoonotic events. Phylogenetic analysis of H5N1 HPAIV isolates from the outbreak among poultry in Ghana, revealed that H5N1 HPAIV isolated from chicken in Ghana in 2015 belonged to the WHO clade 2.3.2.1c, which were reassortant viruses containing PB2 proteins from H9N2 viruses. Ghanaian isolates were closely related to the outbreak strain from Nigeria with 80-99% amino acid homology. Genetic analysis revealed that Ghanaian strains harboured several amino acid motifs, which were responsible for avian mammalian adaptation. Compared to each other, Ghanaian isolates showed slight differences to each other. *In vitro*, we could show that Ghanaian isolates were highly stable at temperatures of 50°C. Ghanaian isolates preferred binding to sialic acids linked to galactose sugars in an α2,3 linkage, showing specificity for avian-like receptors. NA activity was restricted in Ghanaian isolates, compared to control viruses KAN-1 and R65. Polymerase activity of Ghanaian strains was enhanced (up to 150% for virus isolate from Accra) in mammalian cells. In line with observations from polymerase activity measurements, Ghanaian isolates replicated to high titres in human lung epithelial cells. In a BALB/c mammalian model, we could show that H5N1 HPAIV isolates from Ghana were virulent, causing substantial morbidity and mortality. Viruses replicated to high titres in the lungs of infected mice and induced massive infiltration of alveoli. H5N1 HPAIV isolates from poultry in Ghana in 2015 harboured mutations, which most likely mediated adaptation to BALB/c mice. Consequently, H5N1 HPAIV isolates from Ghana may possess zoonotic potential warranting high vigilance.

The data generated from this thesis is very important for Ghana. During this thesis, we characterized H5N1 HPAIV that infected chicken in Ghana and for the first time published these results. We could show that H5N1 HPAIV exhibited zoonotic potential. Skills gained during this PhD thesis will be crucial in improving pandemic preparedness efforts for Ghana.

## 7.0 Literature


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## 8.0 Curriculum vitae

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**EDUCATION**

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| 1999-2003 | Kwame Nkrumah University of Science and Technology Kumasi, Ghana | BSc Biological Sciences |
| 2006-2009 | Microbiology Department, University of Ghana Medical School University of Ghana, Ghana | M.Phil, Microbiology |

**WORK HISTORY**

October 2014 to December 2018

- Heinrich Pette Institute for Experimental Virology, Hamburg, Germany
- PhD student, Viral Zoonoses - One Health

September 2003 to date

- Noguchi Memorial Institute for Medical Research (Virology Department)
- Principal Research Assistant

**GRANTS/AWARDS**

- Deutsche Akademischer Austauschdienst (DAAD) and Ghana Government grant for a PhD to be awarded in Germany. June 2014 to June 2018
- Young scientist travel grant for the Sixth European Scientific working group on influenza (ESWI) conference, Riga, Latvia. 10$^{th}$ – 13$^{th}$ September, 2017.

## CONFERENCES/PRESENTATIONS

- 26th Annual meeting of the German Society for Virology; Münster Germany, April 6th – 9th, 2016. Talk: H5N1 highly pathogenic avian influenza A virus outbreak among poultry in Ghana in 2015.
- 5th International influenza meeting; Münster Germany, September 25th – 27th 2016. Poster: H5N1 highly pathogenic avian influenza A virus outbreak among poultry in Ghana in 2015.
- The sixth European Scientific Working group on influenza (ESWI) conference; Riga Latvia, September 10th – 13th 2017. Talk: H5N1 highly pathogenic avian influenza A virus outbreak among poultry in Ghana in 2015.
- International Summer School on Structural Biology in infection; Lübeck and Hamburg Germany, August 26th – 31st 2018. Talk: H5N1 highly pathogenic avian influenza A virus outbreak among poultry in Ghana in 2015.

## PUBLICATIONS DURING THIS PHD THESIS

- **Asante IA**, Bertram S, Awuni J, Commey ANO, Aniwa B, Ampofo WK. Highly pathogenic avian influenza A(H5N1) virus among poultry, Ghana, 2015. Emerg Infect Dis. 2016 Dec. http://dx.doi.org/10.3201/eid2212.160639
- Stephanie Stanelle-Bertram, Kerstin Walendy-Gnirß, Thomas Speiseder, Swantje Thiele, **Ivy Asantewaa Asante**, Carola Dreier, Nancy Mounogou Kouassi, Annette Preuß, Gundula Pilnitz Stolze, Ursula Müller, Stefanie Thanisch, Melanie Richter, Robin Scharrenberg, Vanessa Kraus, 6 Ronja Dörk, Lynn Schau, Vanessa Herder, Ingo Gerhauser, Vanessa Maria Pfankuche, Christopher Käufer, Inken Waltl, Thais Moraes, Julie Sellau, Stefan Hoenow, Jonas Schmidt Chanasit, Stephanie Jansen, Benjamin Schattling, Harald Ittrich, Udo Bartsch, Thomas Renné, Ralf Bartenschlager, Petra Arck, Daniel Cadar, Manuel A. Friese, Olli Vapalahti, Hanna Lotter, Sany Benites, Lane Rolling, Martin Gabriel, Wolfgang Baumgärtner, Fabio Morellini, Sabine M. Hölter, Oana Amarie, Helmut Fuchs, Martin Hrabe de Angelis, Wolfgang Löscher, Froylan Calderon de Anda and Gülsah Gabriel. Male offspring born to mildly ZIKV-infected mice are at risk to develop neurocognitive 1 disorders in adulthood. Published in Nature Microbiology: https://doi.org/10.1038/s41564-018-0236-1

## 9.0 Declaration of Oath

I hereby declare, on oath, that I, Ivy Asantewaa Asante, have written the present dissertation myself and have not used any other materials other than the acknowledged resources and aids.

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Signature (Hamburg, 25.09.2018)

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