UNIVERSITÉ DE LORRAINE

Ecole Doctorale BioSE (Biologie-Santé-Environnement)

Thèse

présentée et soutenue publiquement pour l'obtention du titre de

DOCTEUR DE l'UNIVERSITE DE LORRAINE

Mention : « Sciences de la Vie et de la Santé »

par **Chantal Snoeck**

Epidémiologie moléculaire des virus de l'influenza aviaire et de la maladie de Newcastle en Afrique de l'Ouest, en Afrique Centrale et au Luxembourg

Le 14 décembre 2012

Membres du jury :

Rapporteurs :	M. Nikolaus MULLER-LANTZSCH	PU, Universtät des Saarlandes, Homburg, Allemagne
	M. Etienne THIRY	PU, Université de Liège, Liège, Belgique
Examinateurs :	M. Alain LE FAOU	PU, EA 3452, Université de Lorraine, Nancy
	M. Claude P. MULLER	PU, Centre de Recherche Public de la Santé/LNS, Luxembourg, directeur de thèse
Membres invités :	Mme Véronique VENARD	MCU-PH, CHU de Nancy-Brabois, Université de Lorraine, Nancy

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aides à la formation recherche

Fonds National de la Recherche Luxembourg

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List of abbreviations

aa	amino acid
AIV	avian influenza virus
APMV-1	avian paramyxovirus type 1
bp	base pair
BLAST	basic local alignment search tool
C	Celsius or cytosine
cDNA	complementary DNA
cRNA	complementary RNA
Ct	cycle threshold
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dNTP	deoxyribonucleotidetriphosphate
F	fusion
FAO	Food and Agriculture Organization of the United Nations
GTR	general time reversible
H/HA	hemagglutinin
HN	hemagglutinin-neuraminidase
HP	highly pathogenic
HPAI	highly pathogenic avian influenza
HPD	highest posterior density interval
I	invariant or Isoleucine
ICPI	intracerebral pathogenicity index
IVPI	intravenous pathogenicity index
IFN	interferon
kb	kilobase
L	large protein or leucine
LP	low pathogenic
LPAI	low pathogenic avian influenza
M	matrix
MCMC	Markov Chain Monte Carlo
MgCl2	magnesium chloride
MDT	mean death time
mRNA	messenger RNA
N/NA	neuraminidase
NDV	Newcastle disease virus
nm	nanometer
NP	nucleoprotein
NS/NEP	nonstructural/nuclear export protein
nt	nucleotide
OIE	World Organisation for Animal Health (Office International des Epizooties)

P	phosphoprotein or proline
PA	polymerase acid protein
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PB1	polymerase basic protein 1
PB2	polymerase basic protein 2
PPMV-1	pigeon paramyxovirus type 1
RNA	ribonucleic acid
RNP	ribonucleoprotein
RT	reverse transcription
SAα2,3Gal	sialic acid α2,3 linked galactose
SAα2,6Gal	sialic acid α2,6 linked galactose
SPF	specific pathogen free
TMRCA	time to the most recent common ancestor
U	unit
UV	ultraviolet
vRNP	viral ribonucleoprotein
VTM	virus transport medium
WHO	World Health Organization
Γ	gamma

Amino acid code

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

Introduction

Parts of the introduction on avian influenza virus were published as:

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Poultry meat and eggs constitute one of the cheap sources of protein around the world and are important sources of revenue for many families in developing countries. They also constitute an important part of the agricultural sector in developed countries where thousands of birds may be kept by a single farmer. Poultry production is threatened by two main viral diseases: highly pathogenic avian influenza and Newcastle disease, with economic and public health implications worldwide.

Avian influenza has been known at least since the end of the 19th century. Low pathogenic avian influenza viruses are naturally circulating in wild birds and constitute the reservoir for introduction of new viruses or genes into domestic birds or mammalian species. In humans, the introduction of new viruses or new reassortants has contributed to five pandemics since the beginning of the 20th century and to the establishment of seasonal influenza subtypes causing the death of 250 000 to 500 000 people every year despite the availability of effective vaccines.

Outbreaks of Newcastle disease were first reported in poultry from Java, Indonesia, and Newcastle-upon-Tyne in 1926, and the disease is now found worldwide. Newcastle disease virus is enzootic in several parts of the world, especially in Asia and Africa, where it has a colossal impact on the local poultry production that is largely based either on small-scale farming system with poor biosecurity measures or on free-ranging birds.

Both diseases have a huge impact on poultry production worldwide, either directly by causing high mortality rates in infected flocks, or indirectly by the tremendous costs of the control measures, including stamping-out, compensatory payments, trade restrictions, vaccination or by affecting the sustainability of poultry production in developing countries.

1. Avian influenza virus

1.1 Classification and nomenclature

Influenza viruses belong to the family of *Orthomyxoviridae*, which includes the genera of *Influenzavirus* A, B and C; *Thogotovirus* and *Isavirus*. Influenza A viruses are further classified into subtypes based on the antigenicity of their hemagglutinin (HA) and neuraminidase (NA) proteins. There are 17 HA subtypes (H1-H17) and 10 NA (N1-N10) subtypes known [1]. The strain nomenclature describes the type of virus, the host, the geographic origin, the strain number, and the year of detection, followed by the HA and NA subtypes, e.g. A/chicken/Nigeria/OG2/2007 (H5N1).

1.2 Morphology, Genome and Protein Structure

Influenza A viral particles are pleomorphic, but generally spherical with a diameter of 80 to 120 nm (Figure 1A). They are enveloped viruses containing eight negative-sense, single-stranded, segmented RNA molecules, corresponding to eight genes. Both the matrix (M) and non- structural (NS) genes encode for 2 proteins (M1 and M2; NS1 and NEP/NS2 respectively). The lipid membrane of the virus is derived from the host cell and has HA, NA and M2 proteins inserted into, whereas the M1 protein underlies this envelope (Figure 1B). The core of the virus particle consists of ribonucleoprotein (RNP) complexes composed of viral RNA segments coated with nucleoprotein (NP) and is associated with the polymerase complex (PB1 polymerase basic 1; PB2 polymerase basic 2; and PA polymerase acid). The non-structural protein (NS1) is a multifunctional protein antagonizing the host cell antiviral response [2; 3]. The nuclear export protein (NEP/NS2) is also associated with the viral RNPs (vRNPs) and M1 protein [4].

Recently, new proteins produced by some influenza A strains have been described. PB1-F2 and PB1-N40 are encoded by alternative open reading frames in the PB1 gene [5]. PB1-F2 localizes in the mitochondria, inducing apoptosis in certain cell types, and in the nucleus where it may influence polymerase activity. However, the function of PB1-N40 has not been elucidated yet [6]. PA-X, encoded by an alternative open reading frame in the PA gene, modulates the host immune response [7].

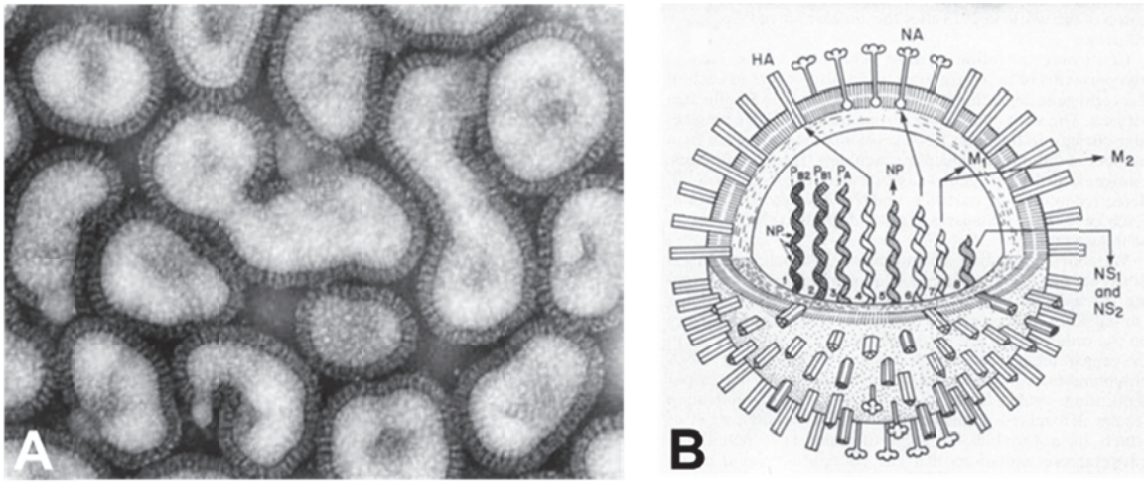

Figure 1. A. Electron micrograph of negatively stained purified influenza A virus particles (G. Murti [8]); B. Schematic representation of an influenza A virus particle [9].

1.3 Viral Replication

Host cell infection starts with binding of viral HA to neuraminic acids on the cell surface followed by endocytosis of the virus. Endosomal acidification induces the fusion of the viral envelope with the endosomal membrane and the release of the vRNPs into the cytoplasm (Figure 2). Those vRNPs migrate to the nucleus, where they act as templates for transcription. In the nucleus, the negative-sense viral RNA (vRNA) is transcribed into mRNA allowing the synthesis of viral proteins. HA and NA proteins are glycosylated in the rough endoplasmic reticulum and transported to the cell surface by the exocytic pathway together with M2. The vRNA is replicated through a positive-sense intermediate, the complementary RNA (cRNA) which in turn is used to produce more vRNA. RNP particles assemble and migrate to areas of the plasma membrane containing the integrated HA, NA, and M2 proteins. Budding of complete viral particles is an active process and mediated by the enzymatic activity of NA, that removes sialic acids from the surface of the host cell [1; 8].

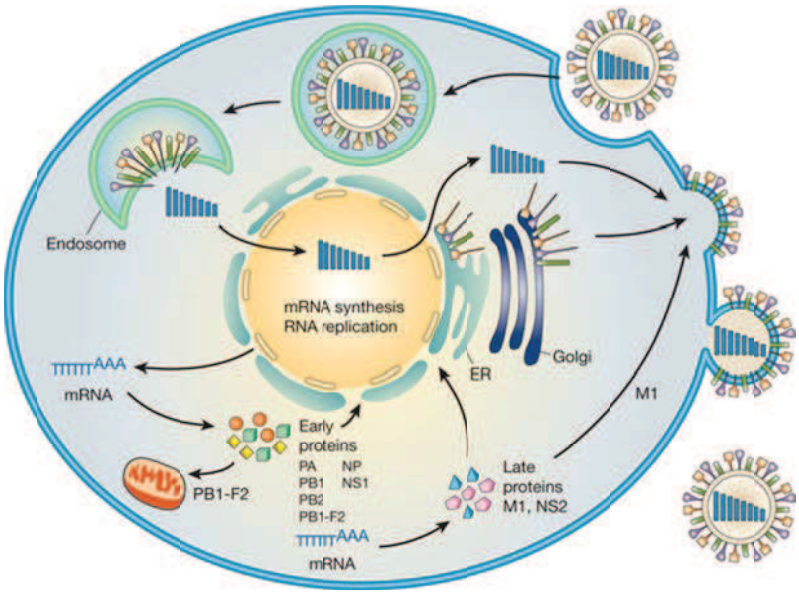

Figure 2. Schematic representation of influenza virus life cycle [10]. After endocytosis mediated by the binding of the HA protein to the cell surface receptor, fusion of the endosomal and viral membranes is promoted by the pH change. Viral RNP particles are released in the cytoplasm, vRNA particles migrate to the nucleus where replication and transcription take place.

1.4 Pathogenesis

1.4.1 Pathotypes

Avian influenza viruses (AIV) can be separated into low (LP or LPAI) or highly pathogenic (HP or HPAI) strains. Most LPAI viruses are asymptomatic in many species of wild birds, or cause mild respiratory symptoms [11]. However, in domestic birds, LPAI strains have been shown to cause mild to severe respiratory signs such as coughing, sneezing, rales, rattles, excessive lacrimation, decreased egg production, ruffled feathers, depression, decreased feed and water consumption, and diarrhea [8].

In contrast, the disease induced by HPAI virus strains, historically been called “fowl plague”, causes severe illness and high mortality rates. After an incubation period of 1-7 days, these strains often cause sudden death without prodromal symptoms. Morbidity and mortality often reach 90-100% within a few days. Birds that survive for 48 hours develop respiratory distress, lacrimation, edema of the head and neck, sinusitis, subcutaneous hemorrhage with cyanosis of the comb, wattles and feet and a comatosis state. Extensive necrotic hemorrhagic lesions, interstitial pneumonia with edema and nephritis were reported at necropsy [12-14]. During the Qinghai Lake outbreak, neurological and gastrointestinal symptoms were reported

in many wild bird species. In addition, extensive pneumonia, myocardial degeneration, focal hepatitis, pancreatic necrosis, were also found in domestic geese and ducks [15].

Pathotyping is essential to contain the spread of HPAI. The conventional method requires experimental intravenous inoculation of infective allantoic fluid into 6-week old Specific Pathogen Free (SPF) chickens. A virus is classified as highly pathogenic if it has an intravenous pathogenicity index (IVPI) greater than 1.2 or if it induces at least 75% mortality in 4-8 week old chickens. Viruses that do not fulfil these criteria should be sequenced to exclude the presence of multiple basic amino acids motifs at the proteolytic cleavage site of the HA protein. If the motif is similar to those of other HPAI isolates, the strain is considered as highly pathogenic [16].

1.4.2 The main molecular determinant of pathogenicity: the HA protein

Pathogenesis and tissue tropism is largely, but not exclusively, determined by the nature of the HA protein and its cleavage site. In the host cell, the HA protein is synthesized as a precursor HA0 which is proteolytically cleaved into HA1 and HA2 subunits. The HA0 precursor of low pathogenic viruses is cleaved by trypsin-like proteases present in the respiratory or intestinal tract [17-20]. In ducks LPAI viruses replicate mainly in the intestinal tract, but also in the lower and upper respiratory tract. Typically the infection of ducks with most strains is asymptomatic [21-23]. In contrast, HA proteins of highly pathogenic viruses possess multiple basic residues at the carboxyl terminus in the subunit HA1, a characteristic of H5 and H7 viruses only (Table 1). This site can be cleaved by ubiquitous cellular proteases (e.g. furin-like proteases) [24; 25]. Thus the virus can cause systemic infections, including the central nervous system involvement, leading to death [26; 27].

1.4.3 Host range restriction

The host species restriction of influenza A viruses is determined by multiple factors including the receptor-binding specificity of the HA protein. Whereas most avian viruses have high binding affinity for sialic acid α2,3 linked galactose (SAα2,3Gal), human influenza viruses bind preferentially to SAα2,6Gal [28]. In humans SAα2,6Gal oligosaccharides are more frequent on non-ciliated epithelial cells of the upper respiratory tract, while SA2,3αGal oligosaccharides are present on ciliated cells of the lower respiratory tract [29; 30]. Receptor distribution in the respiratory tract may explain why humans are easily infected by human strains, whereas infections with avian viruses, probably requiring higher doses, are relatively

rare. In the avian host, e.g. in ducks, SAα2,3Gal are found on epithelial cells of the intestine, where AIV replicates preferentially [31]. Receptor specificity is determined by the amino acids (aa) that form the receptor binding pocket. Glutamine in position 226 (Q226) dictates a preferential binding to sialic acid SAα2,3Gal oligosaccharides present on avian epithelial cells [32]. Mutations in this position (Q226L) of HA allow binding to SAα2,6Gal receptor types of mammalian cells, enhance viral replication in the upper respiratory tract and facilitate transmission to humans. In addition, the number and location of glycosylation sites play a role in virus-host interactions [33].

The NA protein is also involved in host range restriction and pathogenicity [34; 35]. NA activity of some avian viruses is more resistant to the low pH of the upper digestive tract than that of human- or swine-derived NA [36]. NA promotes viral spread within the respiratory tract by cleavage of sialic acids in the mucus [37].

1.4.4 Other determinants of pathogenicity

Normally, avian viruses have glutamic acid in position 627 (E627) of PB2. A lysine at this position favours replication in mammalian cells, correlates with reduced host defence and higher mortality in mice [38-40]. This mutation was not only found increasingly in H5N1 viruses that infected humans since 2001, but also in all subclade 2.2 strains and in all human influenza strains [40-42]. Other aa changes in PB2, PB1 and PA also interact with mammalian adaptation and virulence of HPAI viruses [43-45]. For example, a specific alteration in the protein PB1-F2 (N66S) of HPAI (H5N1) showed increased pathogenicity in mice [46].

NS1 protein is also involved in viral pathogenicity by limiting host cell responses on multiple levels [2; 47-49]. Notably it inhibits both IFN-α/β production and antiviral effects of IFN-induced proteins [48; 50; 51]. Moreover, NS1 inhibits polyadenylation of cellular mRNA, preventing thereby its nuclear export. In parallel, translation of viral mRNA is enhanced by NS1 in the cytoplasm [52], whereas cellular proteins (whose cytoplasmic concentration is kept low by NS1) are poorly translated. This mechanism contributes to reduced host antiviral response by NS1. Remarkably, NS1 proteins of the first HPAI (H5N1) viruses conferred resistance to antiviral effects of IFN, while inducing high levels of proinflammatory cytokines, such as tumor necrosis factor-α and IFN-β [53-55].

1.4.5 Emergence of highly pathogenic viruses

The factors contributing to emergence of HPAI viruses are not clearly understood. However, the current theory is that HPAI viruses emerge only during extensive circulation and adaptation of LPAI viruses in poultry [56]. This hypothesis is based on observations of (i) phylogenetic sublineages of avian influenza including both HPAI and LPAI viruses [57; 58], (ii) *in vitro* selection of a highly pathogenic virus from a low pathogenic virus [15], as well as (iii) on low pathogenic precursor viruses in wild birds that become highly pathogenic after introduction and circulation in poultry, as was observed during the Italian H7N1 outbreak in 1999-2000 [59; 60] and other outbreaks [61-64]. Although the main reservoirs of AIV infections in poultry are infected wild birds, transmission is not a one-way street: wild birds may also become infected by poultry, for example by feeding on infected carcasses [65; 66].

Table 1. Examples of cleavage site sequence of the HA protein of LPAI and HPAI H5 and H7 viruses.

Viral strain	Subtype	Pathotype	HA cleavage site sequence
Typical LPAI H5 viruses			*PQRETR*GLF*
A/turkey/England/91	H5N1	HPAI	PQRKRKTR*GLF
A/tern/south Africa/61	H5N3	HPAI	PQRETRRQKR*GLF
A/chicken/Puebla/8623-607/94	H5N2	HPAI	PQRKRKTR*GLF
A/chicken/Queretaro/14588-19/95	H5N2	HPAI	PQRKRKRKTR*GLF
A/goose/Guangdong/1/1996	H5N1	HPAI	PQRERRRKKR*GLF
Typical LPAI H7 viruses			*PEIPKTR*GLF*
A/turkey/Italy/4580/1999	H7N1	HPAI	PEIPKGSRVRR*GLF
A/chicken/Chakwal/NARC-46/2003	H7N3	HPAI	PETPKRRKR*GLF
A/chicken/Chile/176822/02	H7N3	HPAI	PEKPKTCSPLSRCRETR*GLF
A/chicken/Netherlands/2586/2003	H7N7	HPAI	PEIPKRRRR*GLF

Several mechanisms have been proposed to explain the changes seen in the cleavage site sequence of the HA protein, allowing an increased pathogenicity from LPAI to HPAI viruses (Table 1): i) non-synonymous mutations resulting in the substitution of a non-basic into a basic amino acid [67]; ii) insertions of basic amino acids by duplication resulting from a slippage of the polymerase [67; 68]; iii) insertions of basic and non-basic amino acid stretches of unknown source [69]; iv) recombinations resulting in the insertion of long amino acid stretches from another gene, for instance the NP [70] or M gene [71].

These mechanisms are not mutually exclusive and usually more than one mechanism sequentially contribute to the generation of HPAI viruses. For instance, non-synonymous mutations leading to the change of a non-basic into a basic amino acid can be followed by the duplicaton of the triplet coding for basic amino acids [68]. During the HPAI H7N3 virus outbreaks in Chile, viruses with the cleavage site PEKPKTCSPLSRCR**K**TR*GLF probably evolved from those with the cleavage site motif PEKPKTCSPLSRCR**E**TR*GLF by E to K substitution at the position -3 of the cleavage site [70].

1.4.6 Antigenic drift and antigenic shift

Due to the lack of proof-reading activity of RNA polymerases, mutations occur more often in RNA than in DNA viruses [72]. Mutations introduced in HA and NA proteins tend to modify antibody binding sites, leading to antigenic drift. When a cell is co-infected with two different influenza A strains, viruses can exchange or reassort gene segments. The exchange of HA and NA segments can result in major antigenic changes and immune escape by a process referred to as antigenic shift. Co-infection with viruses of different species may also contribute to the production of new reassortant viruses [73].

1.5 Epidemiology

1.5.1 Influenza A virus reservoir

Wild birds, and in particular water birds, are the natural reservoir of low pathogenic avian influenza viruses (Figure 3) and all H1 to H16 and N1 to N9 subtypes are circulating in wild birds [9]. Only subtype H17N10 recently described in bats in South America has not been found in wild birds [74]. Migration and aquatic environments are important ecological factors for the spread of avian influenza [9; 75-77]. The aquatic environment supports efficient short-range virus transmission by the faecal-oral route [9]. Moreover, during the dry season wild waterfowls are attracted by irrigated wetlands, thus increasing the chances of viral transmission to and from local poultry [78]. Influenza viruses are disseminated over long distances by migratory birds. High densities of mixed bird species at stopover and migration sites promote intra- and interspecies virus transmission and reassortments [77; 79]. Probably due to limited interactions between migratory birds that mainly follow north-south flyways, influenza viruses circulating in wild birds have evolved into two lineages, Eurasian and American [77].

Influenza A viruses can infect a broad range of birds and mammals, including humans and pigs, and sporadic transmission to other hosts resulted in transient or permanent lineages. Poultry (and humans) are at risk whenever wild and domestic birds intermingle [80; 81]. Pigs are often considered as "mixing vessels" that could generate reassortant influenza A viruses because they possess both types of sialic acid respectively preferred by human and avian viruses [9]. Thus, birds are a primary genetic reservoir of influenza viruses, also for mammalian species, including humans [79].

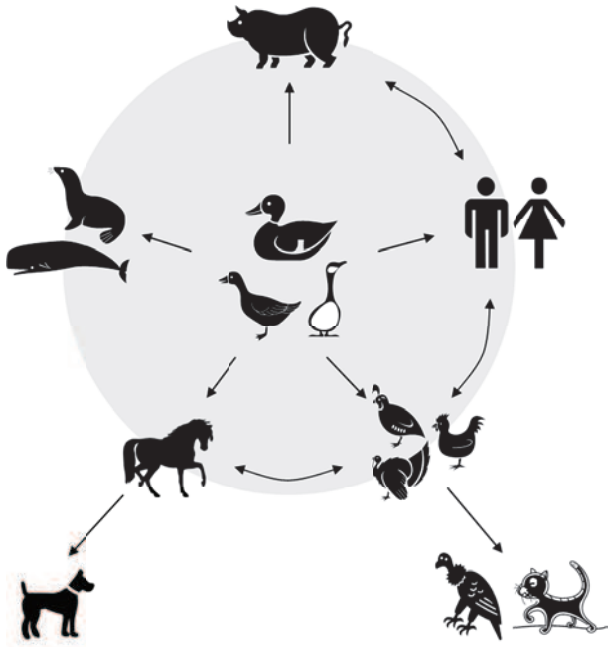

Figure 3. Influenza A virus reservoir (adapted from [9]). Wild birds host all HA and NA combinations discovered so far, except the H17N10 subtype recently found in bats, and can transmit viruses to various animals, including seals, whales, horses, poultry and human. When a virus adapts to sustained transmissions within the new hosts, a new lineage is formed.

1.5.2 LPAI virus outbreaks

LPAI viruses are mainly resident in wild birds but some also infect domestic birds. LPAI H5 and H7 subtypes generate most concern because of their potential to mutate into HPAI strains and therefore are notifiable influenza A viruses. All outbreaks in poultry have economic implications, because of the loss of productivity, the need for large vaccination campaigns or culling. Recently, LPAI H5N2 (Japan, 2005 [82] and Italy, 2005 [83]), H7N1 (Italy, 1999-2001 [84]), H7N2 (USA, 2002 [85]; UK, 2007) and H7N3 (Italy, 2002-2003 [83]; UK, 2006 [86]) viruses were responsible for several outbreaks. Also other non-H5 or H7 subtypes

caused large outbreaks such as H6N1 in Asia [87], H6N2 in South-Africa [88] or in the USA [89].

In the mid-nineties, H9N2 viruses became widespread in poultry in Asia. They also caused several outbreaks in Europe, South Africa, the USA and the Middle East [56] and are now considered enzootic in the Middle East and Asia. Although H9N2 viruses do not have a polybasic cleavage site in the HA, their ability to cause severe respiratory distress, high morbidity and mortality and a drop in egg productivity, depending on co-infections, represent another threat to the poultry industry [90]. Several cases of poultry-to-human H9N2 virus transmission have also been reported [91]. Surprisingly, a large percentage of H9N2 viruses circulating in poultry have a human virus-like receptor specificity (Q226L substitution in HA protein) [56; 92-94]. The co-circulation of H9N2 with other influenza subtypes, paired with their affinity for SAα2,6Gal type receptors, represent a dangerous breeding ground for yet another panzootic or pandemic virus [56; 92; 94].

1.5.3 HPAI virus outbreaks

Besides the Asian HPAI H5N1 virus, more than 30 other HPAI outbreaks, caused by several H5 or H7 genotypes, occurred since the first confirmed HPAI outbreak in 1959 in Scotland [95; 96]. Supposedly all H5 and H7 strains have the possibility to become highly pathogenic in poultry. However, the majority of the HPAI outbreaks were caused by H5N1, H5N2, H7N3 and H7N7 viruses, while H5N3 (1963, South Africa), H5N8 (1963, Ireland), H5N9 (1966, USA), H7N1 (1999-2000, Italy), and H7N4 (1997, Australia) subtypes were responsible of single outbreaks [96]. Several of these viruses were responsible for the death or culling of millions of birds, like H5N2 in the USA in 1983-1985 [97], H5N2 in Mexico in 1994-1995 [98], three H7N3 outbreaks in Pakistan in 1994-1995 [99], 2000 and 2003-2004 [100], H7N1 in Italy in 1999-2000 [59], H7N3 in Chile in 2002 [101], H7N7 in the Netherlands in 2003 [102]; H7N3 in Canada in 2004 [103]. The frequency of HPAI outbreaks seems to increase with time although this could be biased by improved disease reporting.

1.5.4 Chronology of HPAI H5N1 virus outbreaks

First wave. HPAI H5N1 virus was first isolated in Hong Kong in 1997 from a child with fatal respiratory illness. This was the first of 18 patients that became infected from poultry at live bird markets; six of these patients died [49]. Surveillance and epidemiological studies established that several AIV subtypes, including HPAI H5N1 virus, co-circulated in chickens,

ducks and geese in live bird markets in Hong Kong [104]. The initial outbreaks were associated with a relatively low mortality in chickens, but the mortality in humans was as high as 30%. Intensive poultry culling, starting in December 1997 in markets and farms, contained the outbreaks and no new cases were found until 2000 [105]. This HPAI H5N1 virus probably resulted from a reassortment between a H5N1-like virus (HA gene, A/goose/Guangdong/1/96), a H9N2-like virus (internal genes, A/quail/Hong Kong/G1/97) [106] and/or a H6N1-like virus (NA gene and/or internal genes, A/teal/Hong Kong/W312/97) [107].

Second wave. Poultry culling did not interrupt the continuous circulation of A/goose/Guangdong/1/96-like viruses [108; 109], some of which reassorted with unknown viruses from an aquatic bird reservoir. Several genotypes with distinct internal genes emerged in 2001 and 2002, causing a number of outbreaks in China [110; 111]. One of these genotypes (Z) became dominant in Southern China and eventually differentiated into the distinct H5N1 clades that continue to circulate until today. In February 2003, the first human case since 1997 was reported in Hong Kong. This virus (Z+) was similar to genotype Z but lacked the NA stalk deletion, characteristic of genotype Z strains [112]. During the same year, HPAI H5N1 started to spread to other Asian countries: until the end of 2004, the Republic of Korea, Thailand, Vietnam, Japan, Cambodia, Lao PDR, Indonesia, and Malaysia had experienced H5N1 outbreaks in poultry and on rare occasions the virus was also detected in dead wild birds [113; 114].

Third wave. In 2005, a large HPAI H5N1 virus outbreak affected thousands of waterfowl at Qinghai Lake, an important breeding site for migratory birds in Western China. At least 4 genotypes were detected, but one genotype became dominant [37; 115]. By the end of 2005, this virus was reported from Russia, Kazakhstan, Mongolia, Turkey, Romania, Croatia, Ukraine and Kuwait. This was only the beginning and this third wave eventually spread to more countries in Europe, the Middle East and Africa [114]. Wild birds, especially ducks, may have contributed to this long distance spread, since some species were relatively resistant to HPAI H5N1 virus morbidity [80; 116], and some European countries reported HPAI H5N1 cases in wild birds, without outbreaks in poultry [77].

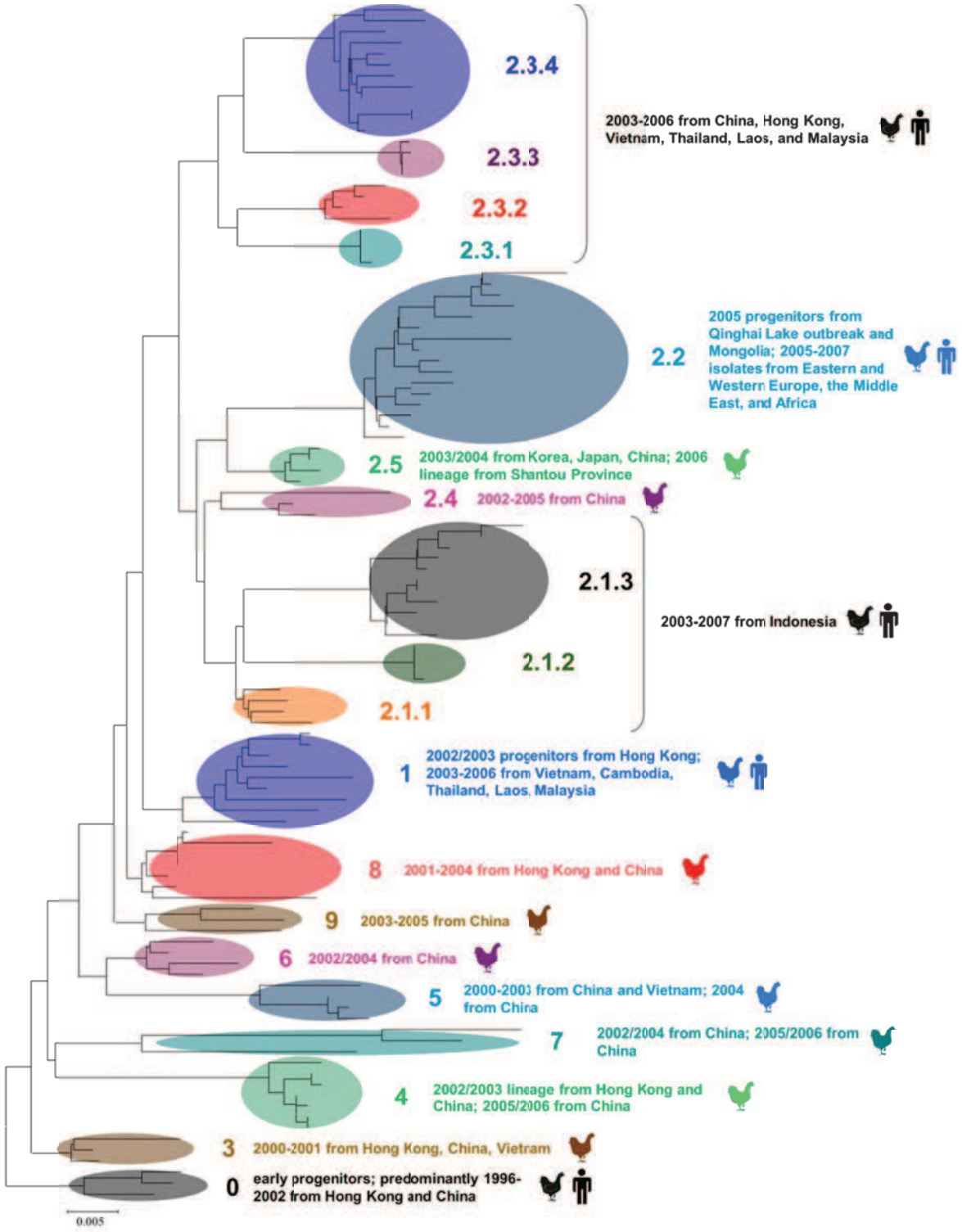

Figure 4. Genetic diversity of HPAI H5N1 virus since its emergence in 1997 (adapted from [117])

During 2005 and later, outbreaks continued in Eastern, Southeastern and Southern Asia, and phylogenetic analyses revealed the co-circulation of several genotypes, named clades. Although various gene constellations resulting from reassortments were observed, the HA gene was still derived from a A/goose/Guangdong/1/96 H5N1-like virus, evolving by genetic drift. This genetic diversity led to the adoption of an international standard nomenclature, which is regularly adapted [117]. Starting with the initial H5N1 strain A/goose/Guangdong/1/96 (clade 0), HPAI H5N1 virus has now evolved into ten major clades (0 to 9) and additional subclades (Figure 4). Clade 1 strains were found in Southeastern and Eastern Asia while Clade 3 to 9 viruses are mainly restricted to Vietnam, Hong Kong and China. Clade 2 strains spread further in Asia, the Middle East, Europe and Africa and diversified into second and third-order groups (Figure 4). All clade 2.2 viruses were derived from early strains (A/bar-headed goose/Qinghai/A1/2005-like strain), transmitted through aquatic birds at Qinghai Lake [117]. The emergence of multiple clades and subclades in Asia reflects the uninterrupted circulation of H5N1 despite culling and vaccination measures [118]. In 2009 eleven Asian countries detected HPAI H5N1 viruses in wild birds, farms and live bird markets [119]. Movement of birds between live bird markets and backyard farms, free ranging ducks acting as an interface between wild and domestic birds, large waterfowl population, legal or illegal bird movements and poor biosafety measures contribute to a favourable breeding ground for influenza A viruses in Asia and beyond [77; 118].

1.5.5 Focus on Africa

A seroprevalence study in sub-Saharan Africa, conducted in commercial poultry in Nigeria between 1999 and 2004, did not detect antibodies against influenza A viruses. Since Nigeria has the largest and the most active poultry industry in that region, this may suggest that at least LPAI viruses did not enzootically circulate in the sub-Saharan region [120]. However, when HPAI H5N1 virus swept from Asia across Russia to Europe, it also reached Africa. The first officially reported case occurred in commercial poultry farms in northern Nigeria in February 2006 [121] although one report based on a single strain suggests that HPAI H5N1 virus was already introduced in 2005 [122]. Soon afterwards, HPAI H5N1 infections were reported throughout most of Nigerian Federal States (http://empres-i.fao.org). The genetic diversity, the timeline, the observed substitution rates, and the phylogenetic relationship suggested that three sublineages (A, B and C) of clade 2.2 were independently introduced

into the country [123; 124]. HA gene sequences clustered with strains found in Europe, Russia and Western China, but were distinct from strains in the rest of China and Southeast Asia [123; 124]. The route of H5N1 introduction into Africa is difficult to establish. While illegal animal trade is not unusual, the presence of several bird sanctuaries along migratory pathways in West Africa are compatible with introductions by migratory birds [125]. Within three months, H5N1 outbreaks were reported in Egypt (sublineage B), Niger (sublineage A), Cameroon, Burkina Faso (sublineage C), Sudan (sublineage C), Côte d'Ivoire (sublineage C) and Djibouti (sublineage B) and all strains were most closely related to viruses found earlier in Nigeria [123; 124; 126; 127] (Figure 5). In Nigeria, the co-circulation of several sublineages led to multiple reassortment events between sublineage A and C viruses. One reassortant AC $_{PB1/HA/NP/NS}$ virus, found only once in 2006, had 4 genes (PB1/HA/NP/NS) from sublineage C and the 4 other genes from sublineage A [128]. In 2007, one AC_{NS} (all genes except the NS gene derived from a sublineage A) [129] and 15 $AC_{HA/NS}$ reassortants were found in several Nigerian states [129; 130]. Interestingly, NS of all reassorted viruses originated from sublineage C viruses, which may reflect an improved fitness and adaptation to the African ecology [129]. In 2007, HPAI H5N1 virus spread also to three other countries, Ghana (sublineage C), Togo (sublineage A) and Benin ($AC_{HA/NS}$ reassortant) [131], probably by bird trade. Reassortant strains from Benin were closely related to Nigerian reassortants from 2007 [131]. In 2008, only four African countries (Nigeria, Togo, Egypt and Benin) reported H5N1 outbreaks [114; 132]. In Northern Nigeria a H5N1 virus, phylogenetically most closely related to European strains, was found [127]. This constitutes the first evidence of a new virus introduction since the first HPAI H5N1 outbreaks in 2006 in Nigeria and suggests that the introduction of HPAI H5N1 in Africa is a rare event. In 2009 only Egypt continued to struggle with outbreaks in domestic poultry [114], because of its high diversity sublineage B, primarily found in Egypt, this subtype has been defined as a third-order clade 2.2.1 [133].

In Egypt, human cases of HPAI H5N1 virus (sublineage B) were reported every year since 2006 (Figure 5). In 2009, 39 of the 52 human cases worldwide were reported from Egypt but the mortality in Egypt dropped from 45% (2006-2008) to 10% (2009) [134], possibly due to viral and/or public health factors. Some Egyptian viruses may also evolve towards a human-like receptor usage (SAα2,6Gal), increasing their replication efficiency in the upper respiratory tract [135]. In addition to Egypt, human cases of HPAI H5N1 occurred also in

Djibouti in 2006 (n=1; sublineage B) [124] and in Nigeria in 2007 (n=1; $AC_{HA/NS}$ reassortant; unpublished data).

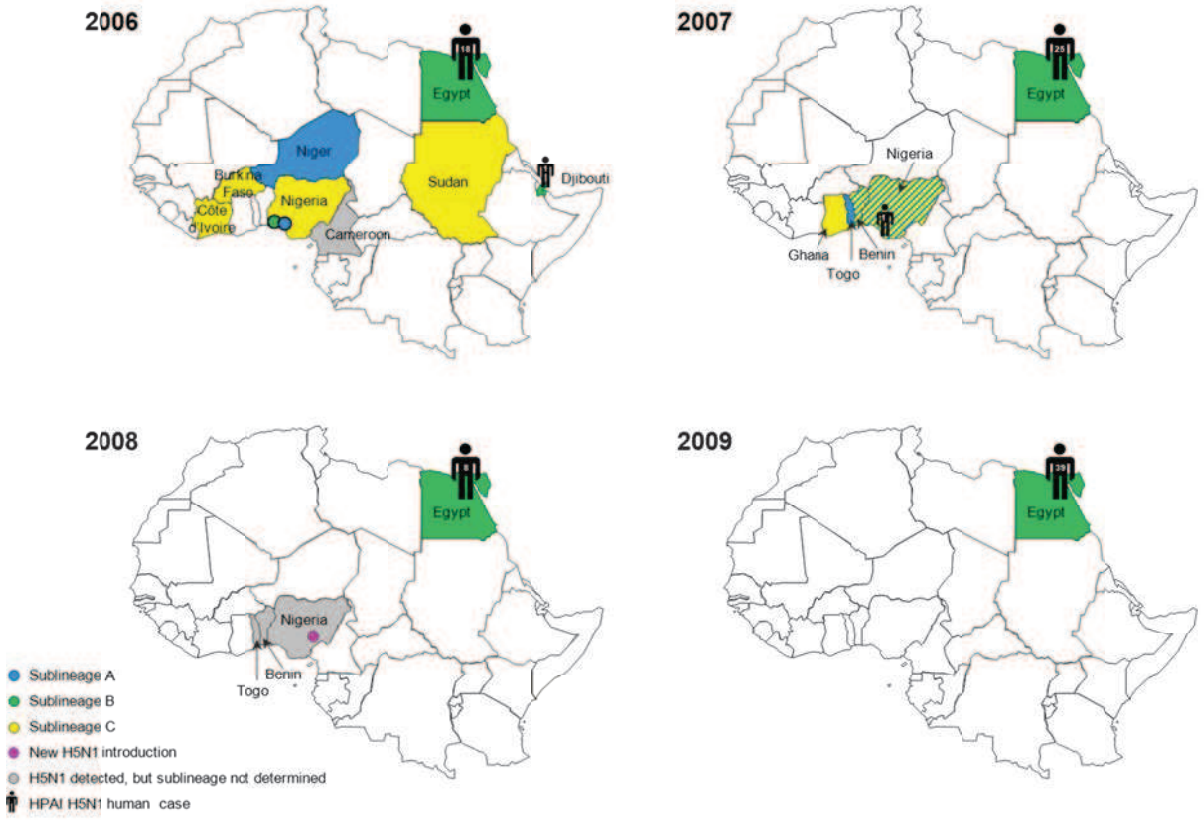

Figure 5. Epidemiology of HPAI H5N1 viruses in Africa since their first introduction in 2006 (based on data collected from http://www.oie.int, http://www.who.int, http://empres-i.fao.org and EpiFlu Database http://epiflu.vital-it.ch).

1.6 Control strategies

Several aspects are important for the control of AIV in poultry and include: education; biosecurity (biocontainment and bioexclusion); diagnostic and surveillance; stamping-out and disinfection; and decreasing host susceptibility by vaccination [8]. The strategies applied may differ depending on the species affected, the type of birds, the viruses involved or simply the country. For instance, layers infected with LPAI viruses (but not H5 or H7) may be allowed to recover from the infection without any further measures. However, stamping-out of flocks infected with LPAI H5 or H7 is more and more common since these viruses may evolve into HPAI viruses after extensive circulation.

The majority of vaccines against AIV used in poultry are inactivated and historically, have been produced by amplifying field LPAI strains in embryonated eggs, followed by inactivation and addition of an adjuvant. In a few occasions, inactivated HPAI strains have also been used [136]. The immunity induced by vaccination is HA subtype specific, therefore an important prerequisite for effective protection is the similarity of the HA of the seed and challenge strain. However, the NA subtype is not important and this characteristic can been used to differentiate the infected from the vaccinated animals (DIVA strategy) by serological testing targeting the NA protein. For instance, a H7N3 seed strain from Pakistan was used as a vaccine during H7N1 outbreaks in Italy in 2000 [137].

The necessity of producing vaccines rapidly and in high quantities when outbreaks are declared has urged the need for alternative vaccine development approaches. Reverse genetics techniques have opened new opportunities in manipulating influenza viruses. To date, vaccines based on recombinant fowl poxvirus or Newcastle disease virus expressing H5 are also licensed [136]. The former has been extensively used during H5N2 outbreaks in Mexico, Guatemala and El Salvador [138].

1.7 Public health implications

1.7.1 Origin of influenza A viruses circulating in humans

All influenza A viruses circulating in humans have their origins in animals. Pandemics occur when a virus with a new HA segment is introduced into the human population by interspecies transmission and/or by reassortment (antigenic shift), whereas yearly epidemics are caused by the constant evolution of the viruses that tend to escape the host immunity (antigenic drift). In 1918, it seems that a wholly avian H1N1 virus spread from birds to humans and caused the so called “Spanish flu” that killed 50 million people worldwide (Figure 6). In 1957, a human/avian reassortant H2N2 virus with new HA, NA and PB1genes lead to the “Asian flu” pandemic. Another human/avian reassortment created the H3N2 virus, leading to the “Hong Kong flu” that started in 1968 [10]. These H3N2 viruses are still circulating today, and co-circulated for more than 30 years with an H1N1 virus that reemerged in 1977 as the so called “Russian flu”. The similarities of the latter with H1N1 viruses from the 1950s suggested that the virus was accidentally released from a laboratory [139].

In April 2009, a novel swine-origin influenza H1N1 virus was identified in specimens from two patients in the United States. A few days later, the same virus was identified in Mexico

and Canada and finally spread all over the world, leading to the most recent human influenza pandemic. This virus exhibited a new combination of genes from American and European swine influenza A viruses [140], suggesting that the virus must have circulated unnoticed in swine. Despite much effort, the region and the timespan of generation of this reasserted virus is still unknown. Several cases of reverse zoonosis, i.e. human-to-turkey [141] and human-to-pig [142] transmissions, have been reported and surveillance has recently shown that swine influenza A viruses and the pandemic H1N1/2009 virus have already reassorted in pigs [143]. In 2011 and 2012, reassorted viruses bearing the matrix segment from pandemic H1N1/2009 and segments from swine triple-reassortant H3N2, H1N1 or H1N2 viruses were found in an increasing number of people in the USA, but in most cases, direct exposure to swine could be confirmed [144].

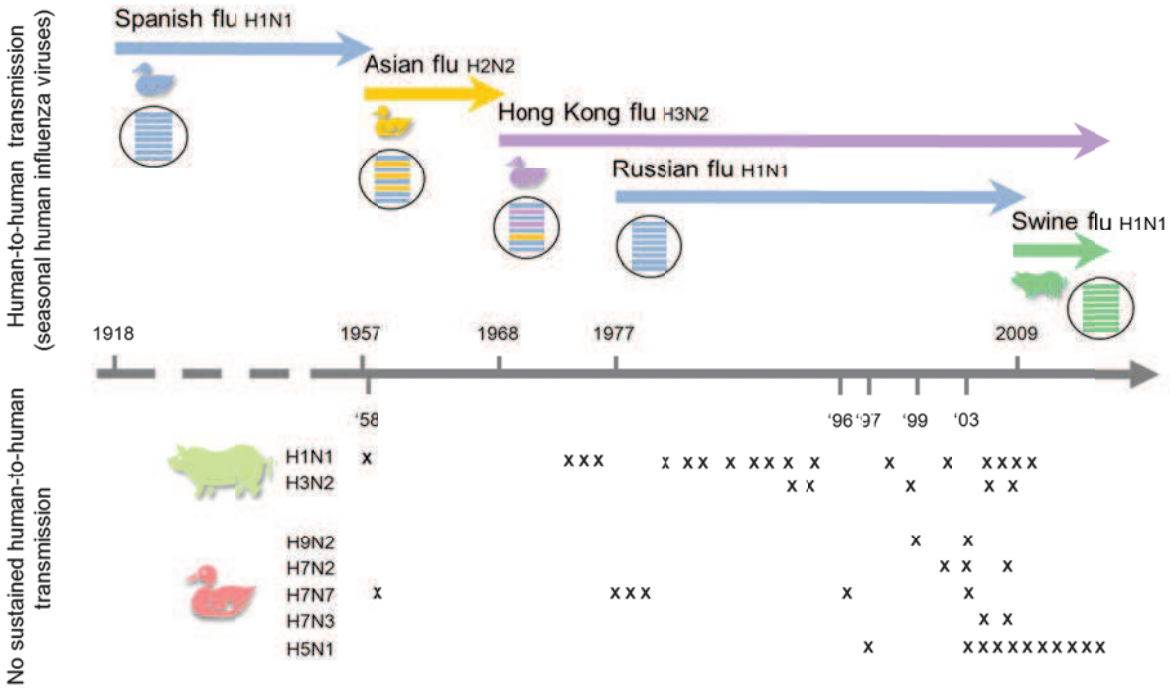

Figure 6. Origin of the influenza A viruses introduced in the human population (adapted from [8; 10]). Viruses with new antigenic properties that were able to transmit easily from human to human have caused pandemics, while transmission of viruses from pigs or birds occur sporadically, especially in people with occupational exposure to farmed animals.

Sporadic transmission of avian or swine viruses to humans have been reported, especially in people with occupational exposure to farmed animals, such as farmers, veterinarians and people involved in animal processing (reviewed by [145; 146]; Figure 6). Human infection with HPAI had rarely occurred before the emergence of HPAI H5N1. One HPAI H7N3 virus

infection was reported in Canada in 2004. Eighty-nine cases were caused by HPAI H7N7 virus transmissions in the Netherlands in 2003, mainly inducing influenza-like illness and conjunctivitis, but one veterinarian died [145]. However, more than 600 HPAI H5N1 virus infections in humans have been confirmed since 2003 and nearly 60% of the cases were fatal [134], leading to the fear that HPAI H5N1 may become the next pandemic virus.

1.7.2 Prevention by vaccination

Annual vaccination with seasonal human influenza vaccines usually containing a H1N1 strain, a H3N2 strain and an influenza B strain, are recommended for risk groups, e.g. elderly and health care personnel. The vaccine composition is updated almost each year to accommodate for antigenic drift.

Since the emergence of HPAI H5N1 virus in Hong Kong, the growing concern of a potential pandemic caused by a highly pathogenic influenza A virus led to a burst of research to develop a vaccine against HPAI H5N1 virus. Several approaches have been developed to comply with the requirements for vaccine production in preparation of a pandemic, such as the necessity of developing a broad long lasting immunity using only low antigen doses. Most strategies include the production of split- or whole-virus inactivated vaccines in embryonated chicken eggs. However, this approach has two major limits: the need and difficulty to have a safe supply of SPF eggs in case of a pandemic and the time required for the production of these vaccines. Cell based vaccines are now encouraged to overcome these problems. The use of adjuvants in vaccine preparations has shown promising results in eliciting strong immune response despite low antigen doses [147]. Live-attenuated strains, already used in seasonal vaccines, offer the advantages of eliciting a rapid and broader immune response and are easier to produce and administrate. They are now investigated as alternatives for the preparation of pandemic vaccines [148-150].

1.7.3 Antivirals

Two classes of antiviral drugs are mainly used in the treatment of influenza A infections. They include neuraminidase inhibitors, namely oseltamivir (Tamiflu®/Roche) and zanamivir (Relenza®/GlaxoSmithKline), or M2 inhibitors, namely amantadine (Symmetrel®/Endo Pharmaceuticals; generic available) or rimantadine (Flumadine®/Forest Pharmaceuticals; generic available) hydrochlorides. Neuraminidase inhibitors interfere with virion realease

from the infected cell and M2 inhibitors block the ion channel activity of M2 protein that is critical for the release of vRNP into the cytoplasm [151]. As a general rule, neuraminidase inhibitors should be preferred to M2 inhibitors because of the increased propensity of influenza viruses to develop resistance against the latter [151].

2. Newcastle disease virus

2.1 Classification and nomenclature

With its non-segmented negative single stranded RNA genome, Newcastle disease virus (NDV), also named Avian Paramyxovirus type-1 (APMV-1) belongs to *Mononegavirales* order. It is classified together with Avian Paramyxovirus types-2 to 11 in the *Avulavirus* genus and the *Paramyxoviridae* family [152]. So far, there is no official nomenclature for NDV strains, although more and more authors tend to use a nomenclature similar to that of influenza viruses, *i.e.* APMV type /host/country/strain number/year of isolation.

2.2 Structural and genomic organization

2.2.1 Virion morphology

Newcastle disease viral particles are pleomorphic, but generally spherical, and measure 100 nm or more (Figure 7). They contain a lipid envelope derived from the host cell plasma membrane. Two glycosylated proteins are inserted into the envelope and form projections on the outer surface of the virion: the fusion (F) and hemagglutinin-neuraminidase (HN) proteins. The matrix (M) protein lies underneath the envelope. The nucleocapsid consists of a single RNA particle and the replication complex includes the nucleoprotein (NP), the phosphoprotein (P) and large polymerase protein (L) (Figure 7).

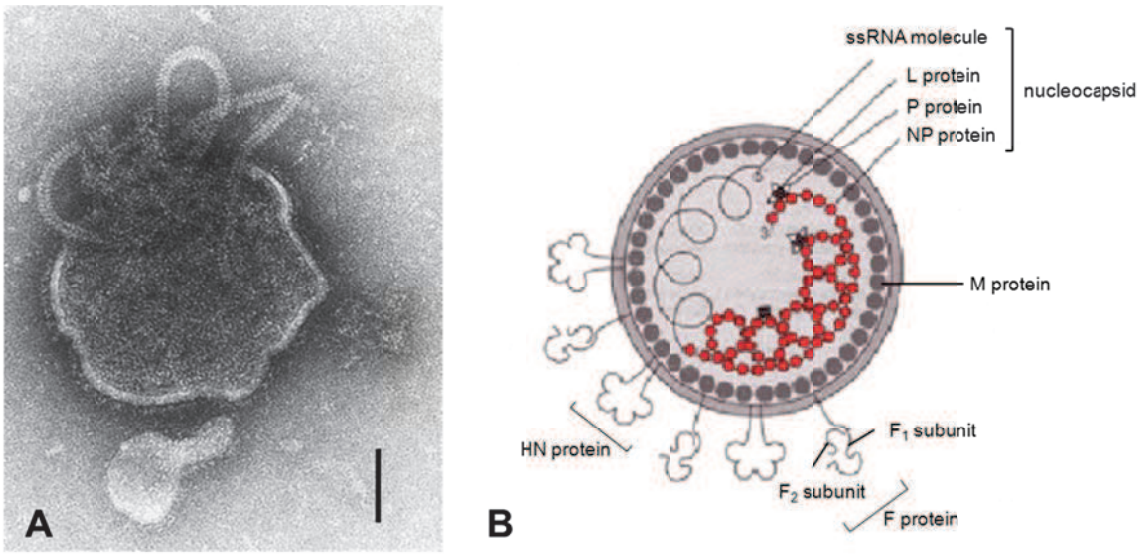

Figure 7. A. Electron micrograph of partially disrupted Newcastle disease virus particle with nucleocapsid emerging, bar = 100 nm (Collins; [153]). B. Schematic representation of a Newcastle disease virus particle (adapted from http://www.brandeis.edu/wanghlab/technologies/apps/newcastle.html)

2.2.2 Genome

Initially, all NDV genomes were thought to be 15 186 nucleotides long [154], but genomes containing 15 192 and 15 198 nucleotides also exist due to different types of nucleotide insertions (Table 4). Genomes of 15 192 nucleotides have an insert of 6 nucleotides in the 5' non-coding region of the NP gene [155] while a 12 nucleotide insertion is found in the P gene in 15 196 nucleotide long genomes [156]. The 6 nt insert seems to slightly increase NDV replication and virulence while the 12 nt insert attenuates the virus [157]. The genome sizes are consistent with the "rule of six" of the members of the *Paramyxovirinae* subfamily, which postulates that RNA molecules must be have a multiple of 6 nucleotides to be encapsidated by NP proteins that bind to exactly 6 nucleotides for efficient replication [158].

The genome is composed of 6 genes arranged in the order 3'-NP-P-M-F-HN-L-5' and encodes for the 6 major proteins. However the P mRNA is edited during transcription by adding 1 or 2 non-templated G residues at position 484, resulting in the transcription of mRNAs with +1 or +2 frameshifts encoding for the V and W proteins [159].

2.2.3 Viral proteins

The NP protein (489 aa) binds to negative (genome) and positive (antigenome) RNA species to protect them from nuclease digestion. Specific binding of NP with vRNA is mediated by

NP-P interactions. The P protein (395 aa) also serves as a bridge between the NP-coated RNA and the L protein which then form the RNP complex. The L protein (2204 aa) is a RNA-dependent RNA polymerase and is thus implicated in viral mRNA synthesis, RNA replication but also in post-transcriptional modifications such as mRNA capping, polyadenylation, methylation of mRNA as well as in RNA stability [160]. The M protein (364 aa) lies underneath the envelope. It interacts with the RNP complex, the lipid envelope, F and HN proteins to assemble viral particles and thus plays an essential role in the budding of new virions [161].

The F protein is a homotrimer that mediates fusion of the viral particle with the plasma cell membrane after conformational change promoted by the HN protein. The F protein, when expressed at the surface of an infected cell, can also mediate its fusion with the neighbouring cells to form multinucleated cells called syncytia [162]. The F protein is synthesized as a precursor F0 (553 aa) that needs to be cleaved into F_1 and F_2 subunits to be active. The HN protein is a tetramer implicated in both the attachment of the viral particle to the cell receptor and the release of progeny virions from the surface of the infected cell through its neuraminidase activity [162]. The majority of NDV strains have a 571 aa long HN protein but longer isoforms (572, 577, 578, 580, 585 or 616 aa) can also be produced depending on the position of the stop codon. The V protein (239 aa) is an alternative product of P gene. It is a minor structural protein of the virion and is found in small amounts associated with vRNP. It is also a virulence factor able to alter the IFN response [163]. The second alternative product of P gene, the W protein, is predicted to be 221 aa long. But the protein itself was never found although W mRNA can be detected in very little amount in infected cells and its role is so far unknown [157].

2.3 Viral replication

After adsorption of the virus to the cell surface receptor mediated by the HN protein, the viral membrane fuses with cell plasma membrane and the vRNP are released in the cytoplasm (Figure 8). Entry of paramyxoviruses in host cells does not require endocytosis and endosomal acidification for releasing the RNP particles, contrary to influenza viruses.

In the early phase of the replication cycle called the “primary transcription”, the vRNA serves as template for the transcription of mRNAs. The 3’ leader region contains the promoter and drives the transcription initiation. Gene-start (located in 3’ of each gene) and gene-stop (5’)

transcriptional control sequences define the regions transcribed by the polymerase complex (P-L). Then mRNAs are capped, methylated and polyadenylated. Not every viral polymerase that stopped at the end of a gene reinitiates transcription of the following gene, leading to a gradient of abundance of mRNA according to the distance of the gene to the 3' end. Therefore, NP mRNA is the most abundant and L mRNA is the least abundant [162].

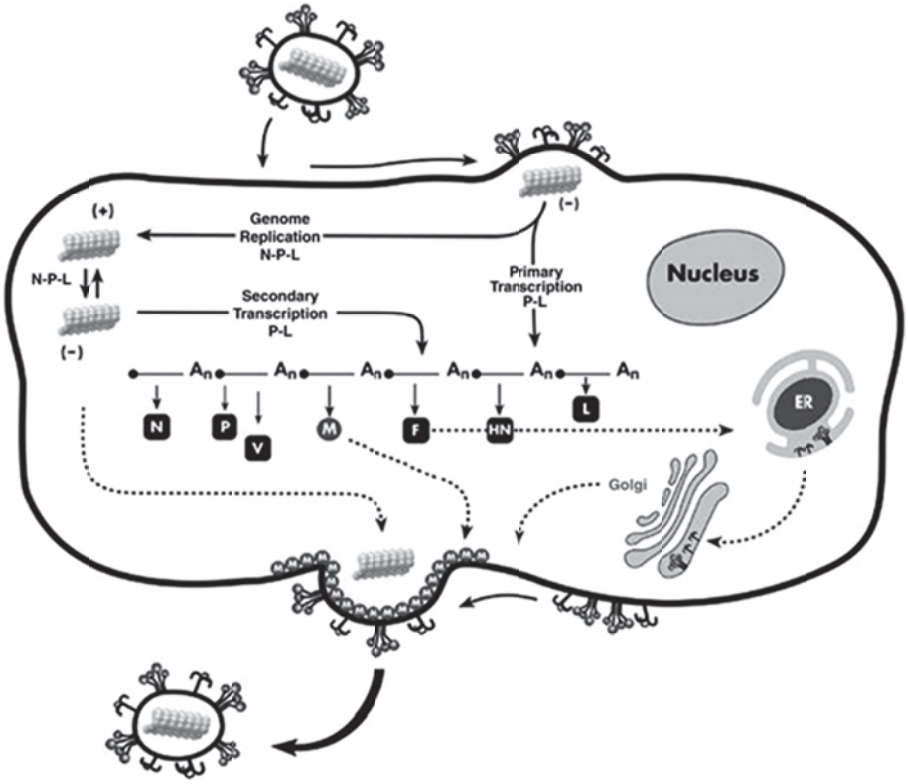

Figure 8. Replication cycle of NDV (adapted from [162])

After translation of the primary mRNA transcripts and accumulation of viral proteins in the cytoplasm, a switch from transcription to replication occurs resulting in the polymerase ignoring gene-stop signals and producing full-length +RNA, called antigenome. Antigenome molecules serve as template for producing genomic –RNA which can be transcribed into viral mRNA ("secondary transcription" phase), replicated or directly incorporated into progeny virions.

Genome and antigenome RNA particles are directly coated with NP subunits and form helical structures. NP coated negative sense RNA particles associate with P-L and are transported to the plasma membrane, where M proteins also accumulate. Viral glycoproteins are transported to the cell plasma membrane by the secretory pathway after maturation in the Golgi

apparatus. Progeny virions are released from infected cells by budding and the HN cleaves the budding virions from the cell surface through its neuraminidase activity [162].

2.4 Pathogenesis

2.4.1 Pathotypes and clinical symptoms associated

NDV strains exhibit a great variability in pathogenicity and have therefore been grouped into five pathotypes on the basis of the clinical symptoms induced in infected chickens. Velogenic viscerotropic NDV is a highly pathogenic form causing hemorrhagic intestinal lesions and necrosis of the lymphoid tissues [164]. Mortality often reaches 100%. The main clinical signs include conjunctivitis, anorexia, ruffled plumage, prostration, green diarrhea, muscular tremors, paralysis and torticollis but high mortality rates without clinical signs may also happen, especially in young birds [153; 165]. Velogenic neurotropic strains usually cause high morbidity (up to 100%) but lower mortality rate (around 50%). They induce mainly neurologic symptoms such as head twitch, tremors, opisthotonus, hemiparesis, paralysis as well as a drop in egg production but gut lesions and diarrhea are usually absent [153; 165]. Mesogenic strains usually cause respiratory signs, a prolonged drop in egg production and occasionally nervous problems, but low mortality. Lentogenic viruses cause subclinical respiratory infection in adults but may lead to more severe disease in susceptible young birds. Finally, asymptomatic enteric NDV is a form that usually causes subclinical enteric infection, without respiratory signs [153].

Table 2. Pathotype designation based on standard pathogenicity tests

Pathotype	MDT*	ICPI
Lentogenic	> 90	< 0.7
Mesogenic	60-90	0.7-1.5
Velogenic	< 90	> 1.5

*MDT is measured in hours

Several laboratory tests can be performed to determine the pathogenicity of NDV strains (Table 2), namely MDT (mean death time), IVPI (intravenous pathogenicity index) or ICPI (intracerebral pathogenicity index) but a definitive pathogenicity assessment must be achieved by ICPI. Newcastle disease, notifiable to the OIE, is defined as an infection with a

NDV strain having an ICPI ≥ 0.7 or a multiple basic amino acid cleavage site of the F protein [166].

2.4.2 Molecular determinants of pathogenicity

It has long been accepted that the main determinant of NDV pathogenicity resides in the F protein. During the replication cycle, the fusion protein is translated as a precursor F0 that must be cleaved by host cell proteases into two subunits (F_1 and F_2) for viral particles to be infectious [167]. Similarly to influenza viruses, the activation of the F0 precursor is dependent on the amino acid motif present at the cleavage site. Consensus sequence of velogenic and mesogenic strains is ^{112}R/K-R-Q-R/K-R*F^{117}, while lentogenic strains exhibit a ^{112}G/E-R/K-Q-G/E-R*L^{117} cleavage site [168] (Table 3). In most virulent viruses, additional basic amino acids allow the cleavage of the F0 precursor by proteases, most likely furin-like enzymes, present in a wide range of cells, permitting the replication in many tissues and organs resulting in fatal systemic infection [169]. On the other hand, the F0 precursor of lentogenic is recognized and cleaved by trypsin-like proteases, restricting the virus replication to the respiratory and digestive tract [170]. Based on the amino acid sequence of the F cleavage site, NDV strains can be classified as virulent or avirulent (Table 3).

Table 3. Various sequences of the fusion protein cleavage site

Strain	Sequence (aa 112 to 117)	Virulence	Lineage
Queensland V4	GKQGR*L	Avirulent	Lineage 1
LaSota	GRQGR*L	Avirulent	Lineage 2
Beaudette	RRQKR*F	Virulent	Lineage 2
Mallard/US(OH)/86-233/1986	EKQGR*L	Avirulent	Lineage 2a
Herts/33	RRQRR*F	Virulent	Lineage 3b
Cormorant/US(MN)/92-40140/1992	RRQKR*F	Virulent	Lineage 3c
Pigeon/Illinois/37397/1987	GRQKR*F	Virulent	Sublineage 4b
Pigeon/South Dakota/486839/2007	RRKKR*F	Virulent	Sublineage 4b
Goose/Jiangsu/JS03/03	RRQKR*F	Virulent	Sublineage 5d
Goose/Alaska/415/1991	ERQER*L	Avirulent	Lineage 6
Duck/China/NDV09-042/2009	EQQER*L	Avirulent	Lineage 6

However, several observations suggest that the F protein cleavage site sequence is not the only virulence factor. Mesogenic strains may have the same cleavage site as velogenic ones but have lower ICPI values. Viruses with a cleavage site typical of virulent strains were found

in healthy migratory ducks in Alaska, and pathogenicity assessment by standard tests showed that these viruses were not virulent for chickens [171] Similarly, sublineage 4b strains isolated from racing pigeons with nervous symptoms before death and with a cleavage site typical of virulent viruses had low ICPI in chickens [172]. Serial experimental passaging in chickens resulted in increased virulence despite no change in the cleavage site [173], but mutations occurring in the P and L genes could explain the increase in virulence [174].

Reverse genetics experiments where a virulent cleavage site was inserted into an avirulent backbone did not result in a complete conversion to a virulent pathotype [175]. Increase in ICPI values of infectious clones after passaging in chicken brains despite no difference neither in the cleavage site sequence nor in other regions of the F gene suggested that mutations might accumulate on other genes [176]. Introduction of the HN gene sequence from a virulent virus into the LaSota strain containing a virulent cleavage site, resulted in higher ICPI and IVPI values compared to the same clone with the HN sequence of the LaSota strain [177]. It has also been suggested that the proteins involved in the replication complex, *i.e.* NP, P and L, of velogenic viruses have an increased activity and contribute to their virulence [178].

By interfering with the IFN response, the V protein also plays a role in the virulence of NDV. It has been shown that Beaudette C strain (mesogenic) had a more efficient IFN antagonist effect than the lentogenic LaSota strain, allowing the mesogenic strain to grow to higher titers [179].

2.4.3 Emergence of virulent viruses

Surveillance and experimental studies have recently provided new insights into the mechanisms underlying the emergence of virulent strains. Two particular cases in Ireland in 1990 and Australia in 1998-2002 have provided field evidence that virulent viruses may arise from avirulent strains by mutations. Outbreaks in layers in two Irish farms were caused by virulent lineage 6viruses related to avirulent strains circulating in chickens in Ireland during the previous years and in wild birds in Europe [180]. It was shown that a minimum of four nucleotide mutations were necessary to pass from ERQER*L to KRQKR*F cleavage site [168], but no direct progenitor was found.

More concrete evidence was obtained in Australia where several isolates from different outbreaks were available. Phylogenetic analyses revealed that the viruses identified during

the outbreaks were more closely related to avirulent lineage 1 strains circulating in Australia, and thus were not imported [181]. In almost every outbreak, progenitor viruses with RRQGR*L and virulent viruses with RRQRR*F cleavage site sequences were circulating [181], suggesting that the evolution from avirulent to virulent occurred independently at the different locations [182]. Intermediate cleavage site sequences RRQRR*L and RRQGR*F, with only one nucleotide mutation away from the RRQRR*F sequence were also found, and two variants RRQGR*L and RRQRR*L were obtained from a single animal. In addition, analyses of quasispecies showed that the isolate with a consensus sequence RRQGR*F contained a mixture of strains with RRQGR*F or RRQRR*F sequence, with the latter being virulent [182].

Transition of an avirulent into a virulent strain was also demonstrated experimentally by passaging a typical waterfowl lineage 6 strain in chickens. After nine air sac passages, the ICPI value increased from 0 to 1.2 and the cleavage site sequence evolved from ERQER*L to KRQKR*L. Additional five intracerebral passages resulted in the generation of viruses with ICPI value of 1.88 with a KRQKR*F sequence [183]. Similarly, passaging of an avirulent lineage 2a strain from a swan in embryonated eggs increased the virulence of the strains, as measured by MDT. After five passages, the cleavage site changed from GKQGR*L to GRQKR*F as a result of five mutations but the MDT value was still indicative of an avirulent strain. After 20 passages, the cleavage site sequence did not change but the MDT decreased from >168h to 83h, typical value of a mesogenic strain. Comparison of the HN protein of the viruses after five and 20 passages showed that the HN protein length decreased from 616 aa, the typical HN length of avirulent strains, to 571 aa, the typical HN length of mesogenic and velogenic strains, due the introduction of a new stop codon, suggesting that the HN length has an important effect on the increased virulence [184].

The emergence of virulent viruses from an avirulent background seems to happen only rarely in the field as only two cases have been reported. However, it is possible that this phenomenon had already occurred in the past [153], giving rise to the virulent NDV lineages known today.

2.5 Epidemiology

2.5.1 Antigenic and genetic variability

Despite the fact that NDV consists of a single serotype, both antigenic and genetic diversity are recognized. Monoclonal antibody panels have been used to detect slight variations in antigenitiy in order to characterize and classify NDV strains [185]. Generally, monoclonal antibody typing and genetic classification based on the F gene by RFLP or sequencing have been shown to give similar results [186-189]. However phylogentic analyses based on a minimum of 250 bp fragment of the F gene [190; 191] is nowadays the most widely used method for classification, and two nomenclature systems co-exist (Table 4). In this thesis, the classification based on Aldous *et al.* [186] was adopted.

Table 4. Correspondence between the two classification nomenclature of NDV and their genome size

Classification based on Ballagy-Pordani *et al.* [187]and Czegledi *et al.* [156]		**Classification based on Aldous *et al.* [186]**	**Genome size**
Class II	Genotype I	Lineage 1	15 186 nt
	Genotype II	Lineage 2	15 186 nt
	Genotype IIa	Lineage 2a	n.d.*
	Genotype III	Lineage 3a	15 186 nt
	Genotype IV	Lineage 3b	15 186 nt
	Genotype V	Lineage 3c	15 192 nt
	Genotype VIII	Lineage 3d	15 192 nt
	Genotype IX	Lineage 3e	15 186 nt
	Genotype X	Lineage 3f	n.d.
	Genotype XI	Lineage 3g	15 192 nt
	Genotype VI a-h	Lineage 4 a-h	15 192 nt
	Genotype VII a-e	Lineage 5 a-e	15 192 nt
Class I	Genotypes 1 to 9	Lineage 6	15 198 nt

* not determined

2.5.2 Host range

Natural or experimental NDV infection has been demonstrated in at least 241 avian species and all domestic bird species (Kalenta and Baldauf, cited by [153]). Since then, many more species have been added to the list and Alexander and Senne therefore concluded that the vast

majority of avian species are susceptible to NDV [153], but the presence of clinical signs may depend on the infected species.

Specific species may serve as reservoir of particular NDV lineages. In particular waterbirds seem to be a natural host of lineage 1, 2a and 6 [192-194]. Poultry population constitutes the most likely reservoir of virulent viruses [193], with few exceptions. Racing and show pigeons are the reservoir of virulent sublineage 4b strains worldwide, but it seems that the viruses have spilled back to feral pigeons and doves in some regions [195-197]. In North America, lineage 3c are enzootic in Double-crested Cormorants [198; 199] and can be transmitted to other species nesting close to their colonies, such as Great Cormorants, Herring Gulls and Black-backed Gulls [200]. Clinical signs including torticollis, paralysis of the legs and wings, tremors, and lack of muscular coordination, particularly affects young birds [200] but adult and older juvenile cormorants seem more resistant to the disease [201]. In Europe, the existence of a wild bird reservoir of lineage 5b strains has been hypothesized after the outbreaks in the UK, Sweden, Denmark and Finland between 1996 and 2005, which were caused by similar strains although a direct epidemiological link was not found [202]. This hypothesis is also supported by the detection of lineage 5b strains in a Goosander in Finland and a cormorant in Denmark [203].

NDV can also naturally infect non avian species such as mice, pigs and humans, and accordingly successful experimental infections of a broad range of mammal species have been reported. For instance, vaccine-like strains such as LaSota and Queensland/V4 were isolated in pigs with influenza-like illness in China, and were attributed to the common use of vaccines [204; 205], but further surveillance studies are needed to understand if these viruses can spread in pigs.

2.5.3 Global epidemiology

The genetic diversity and epidemiology of NDV is rather complex. Altough it seems that lineages have somehow emerged sequentially and that partial regional specificity exists, several lineages usually co-circulate within the same region. In addition, the overview of NDV epidemiology may be biased by a lack of surveillance in some parts of the world, especially before the 1990s and still today in Africa and South America.

Lineage 1. Lineage 1 strains are found worlwide in wild and domestic birds and include principally avirulent viruses replicating primarly in the gut, except for virulent viruses that

caused outbreaks in 1998-2002 in Australia which apparently derived from avirulent strains by point mutations [181; 182]. Derivatives of lineage 1 strains such as chicken/Australia/QueenslandV4/1966 and chicken/N.Ireland/Ulster/1967 are often used as live vaccines.

Lineage 2. Lineage 2 strains, together with lineage 3a and 3b, were responsible for the first panzootic that started in the mid-1920s. Lineage 2 is heterogenous in pathotypes since it contains lentogenic, mesogenic and neurotropic velogenic strains. The widely used avirulent vaccine strains LaSota, VG/GA and B1 belong to lineage 2, as well as the mesogenic vaccines Roakin and Komarov which are used where virulent NDV is endemic [186]. The use of vaccination succeeded in controlling velogenic lineage 2 strains, at least in the USA, and nowadays the majority of lineage 2 strains detected are associated with the use and spread of live vaccines.

Lineage 2a. Lineage 2a was only recently recognized. Viruses belonging to this phylogenetic lineage seem to be naturally circulating in wild migratory fowls in North and South America as well as in the Carribean Islands [192; 194; 206]. One isolate from a chicken from Spain in 1988 also clusters in this lineage, but was previously attributed to lineage 1 [186].

Linage 3. Lineage 3 is divided into several sublineages. The earliest sublineage 3a strain originates from Australia in 1932 and most sublineage 3a strains were isolated before the 1960s. The mesogenic vaccine strain Mukteswar (derived from an Indian isolate) mainly used in Asia belongs to lineage 3a [186] and most, if not all, of the recent sublineage 3a strains found in Asia seems to be derived from the vaccine [207; 208].

The earliest representatives of sublineage 3b date from 1933 (Herts 33) and 1945 (Italien/45) and sublineage 3b became predominant in Western Europe during the 1960s. It was still sporadically found in Sudan in 1972 [186], in Italy until 1987 [189], in Bulgaria until 1982 [188], in Hong Kong in 1989 [186] and in Marocco in 2002 [156].

The second panzootic, which started concomittantly in the USA and in western Europe in 1970, was caused by sublineage 3c strains. The original strains were traced back to infected parrots shipped from South America to the USA [187; 189]. They led to major outbreaks in California from 1971 to 1973 [209], and widely circulated in Europe where they spread south- and eastwards and became predominant. They were still present until the end of the 1980s in several western European countries [188; 189; 210; 211] and persisted at least until 2002 in Serbia [211]. Sublineage 3c strains were also responsible for outbreaks in domestic

birds in Mexico, Honduras and the USA between 1996 and 2006 [212; 213] and are circulating and established in cormorants in the USA and Canada since 1990 [198-200].

The first strain of sublineage 3d was found in Singapore in 1965 [214]. Then this lineage has been sporadically found in China [215], in Japan [216], in Italy [189], in Taiwan [217] and in South Africa [218]. The first sublineage 3e strain dates from 1948 in China and so far sublineage 3e strains have only been occasionally described in China [208; 219; 220]. Recently, 2 new sublineages 3f and 3g have been described. Sublineage 3f strains were so far only reported in Taiwan in 1969 and 1981 [217] and sublineage 3g strains were found in Madagascar in 1992 and 2008 [221].

Lineage 4. Lineage 4 is separated into several sublineages differing in their geographic repartition and spread. During the late 1960s in the Middle East and Greece, the second panzootic was caused by sublineage 4a strains, and since then members of this sublineage have been occasionally reported in the Middle East and Europe [186; 203]. Interestingly, strains distantly related to sublineage 4a were probably responsible for mass mortality in Collared Doves in Italy in winter 2010-2011 and it was suggested that doves may be a reservoir for these particular strains [222].

The third panzootic corresponds with the emergence of sublineage 4b strains in pigeons, also called pigeon paramyxovirus type 1 (PPMV-1), in the late 1970s in the Middle East, but are nowadays commonly found in Asia [208; 223], Europe [203], North and South America [195; 224-226] and South Africa [227]. Originally circulating in racing and show pigeons, they became enzootic in feral pigeons and doves in some countries [195-197]. Sublineages 4b strains have in several occasions been transmitted to domestic birds [197; 203; 228]. They have various degrees of virulence for chickens and virulence may increase after passaging [173; 174; 229].

Most sublineage 4c were described in Saudi Arabia and United Arab Emirates in the 1990s but also occasionally in Italy, Belgium, UK [186] and Japan [216]. Sublineage 4d strains seem to have emerged in the 1970s and were reported in several European countries, such as Bulgaria, Hungary and Germany, during the 1970s and the 1980s [188]. It also circulated in 1995-1996 in Denmark, Austria, Switzerland, and Sweden [230] and is still sporadically reported in Asia and Europe [203; 216].

Sublineage 4e seems to be restricted to a the single isolate Warwick/66 from the 1966 in the UK [187]. Sublineage 4f and 4g were occasionally found in China since the mid-1980s [208;

219] while sublineage 4h is represented by a few Taiwanese strains from 1969 and 1980 to 1984 [217].

Lineage 5. Lineage 5 is also divided into several sublineages and the emergence of these strains is considered by some authors as the fourth panzootic. The earliest sublineage 5a strain originated from Taiwan in 1984 [217] and this sublineage was responsible of severe outbreaks in several European countries (Germany, Belgium, The Netherlands, Italy, Spain) between 1992 and 1996 [190]. This sublineage was also observed in the second part of the 1990s in China [215] and Taiwan [217]. Sublineage 5b strains already circulated in Italy and Bulgaria as early as 1984 but most isolates were described in the 1990s on several continents: in Africa (South Africa, Mozambique, Burundi), in Europe (Ireland, Portugal, Sweden, Bulgaria, Spain, Italy, Turkey), in the Middle East (Pakistan, Iran) and Asia (India, China, Khazakstan) and in South America (Peru) [186; 214]. Several outbreaks caused by sublineage 5b strains were also reported in Western and Northern European countries during the last decade [203]. The earliest representative strain of sublineage 5c was found in Taiwan in 1984. It has also been identified in Czech Republic and Switzerland in 1996 [186], but the majority of strains were described after 1995 in Taiwan and China [186; 208; 231; 232]. Sublineage 5d and sublineage 5e seem to have emerged in the late 1990s in Eastern Asia and they are nowadays responsible for the majority of the outbreaks in China and Taiwan, respectively [207; 208; 219; 231; 233]. Some sublineage 5d strains were also identified in South Africa [218], the Middle East [186] and Malaysia [234]. Several sublineage 5d cases have also occurred in Eastern Europe as a result of westward spread [203].

Lineage 6. Lineage 6 strains are circulating worldwide and wild birds seem to be a reservoir of these avirulent strains. Evidence also suggests that lineage 6 strains were transmitted to domestic poultry from live bird market in the USA and Hong Kong [192; 235]. All strains are avirulent, except one strain that was responsible for outbreaks in poultry in Ireland in 1990 [180].

2.5.4 Focus on Africa

Newcastle disease was first reported in Kenya in 1935 and subsequently in South Africa in 1945 (Kashula *et al.*, cited by [236]), in Madagascar in 1946 [221], in Sudan in 1951 [237], in Nigeria in 1953 (Hills *et al.*, cited by [238]) and Uganda in 1955 [237], but NDV may have been introduced in Africa long before [236]. It is commonly recognized that NDV is enzootic in Africa and constitutes a major threat to the local poultry industry. Seroprevalence studies

performed during the late 1980s-early 1990s showed variable anti-NDV antibody rates: 4.6% in Mauritania, 13.3% in Tanzania, 14% in Niger, 56% to 75% in Benin, 47% to 52% in Cameroon and 72% in Nigeria (reviewed by [239]). Also 957 outbreaks were reported in 31 African countries between 1985 and 1989 [240], but this number is likely to be an underestimate.

However, our current understanding of the circulating strains is still scarce because only few viruses have been characterized antigenetically or genetically. The situation in South Africa is so far the best known, probably due to a longer history of laboratory testing and reporting. Lineage 3d strains were circulating enzootically between 1968 and 2004 [214; 218]. In the 1990s, virulent lineage 2 strains and sublineage 5b were co-circulating and sublineage 5b strains most probably spread from South Africa to Botswana, Zimbabwe and Mozambique [214; 236]. However, sublineage 5b strains from Burundi (2008) were not most closely related to other African strains, suggesting an independent introduction [241]. Sublineage 5d strains were reported for the first time in South Africa in 1999 but were circulating at least until 2006 [236]. They also caused outbreaks in Sudan between 2003 and 2006, but constituted a different introduction from the South African sublineage 5d strains [242]. Outbreaks in pigeons and doves have been reported since the mid-1980s in South Africa and the symptoms were similar to those induced by sublineage 4b strains in European pigeons (Pienaar and Cilliers, cited by [236]). Sublineage 4b was separately introduced at least twice in South Africa, and the viruses were transmitted to chicken flocks during outbreaks in pigeons and doves between 2004 and 2006 [236]. Lineage 4 strains were also detected in Sudan (1985), Kenya (1990) and Tanzania (1995) [186]; lineage 3c in Uganda in 2001 [243] and in Tanzania in 1995 [186]. Virulent lineage 2 strains were also reported in Egypt in 2005 and 2006 [244]. Interestingly, lineage 3g strains which are more closely related to the “old” lineages of NDV have so far only been described in Madagascar, suggesting that viruses have continuously evolved since the introduction of an ancestor decades ago [221].

2.6 Control strategies

The same aspects are important in the control of both AIV and NDV, but more emphasis will be put on one or the other depending on the country. Even in Europe, vaccination policies against NDV may differ. For instance, vaccination is not allowed in Sweden, Finland, Estonia or Luxembourg whereas it is compulsory for chickens and turkeys in the Netherlands and for all commercial birds in Italy [245].

As NDV consists of a single serotype, any vaccine strain should confer a good protection against challenge, given that it is correctly applied. Several seed viruses, typically lentogenic strains, have been used, such as Queensland V4, I-2, Ulster (lineage 1), LaSota, Hitchner B1, VG/GA, clone 30, strain F (lineage 2), or mesogenic strains such as Roakin or Komarov (lineage 2), Mukteswar or strain H (lineage 3a). Due to their higher virulence, mesogenic strains are only used as a boost in regions where NDV is enzootic, mainly in Africa and Asia.

Inactivated and live vaccines are available. They both have advantages and disadvantages and therefore they are usually used in different contexts. Inactivated vaccines must be injected intramuscularly or subcutaneously, hence increasing the costs of vaccination, but are easier to store [153]. Most of the adjuvants used in inactivated vaccines induce tissue damage, therefore they are not suitable for broilers [157]. The main advantage of live vaccines is the possibility of mass application via drinking water or aerosols, but can also be applied to individual animals. Live vaccine strains induce a viral infection and may spread from successfully vaccinated animals to those in the flocks that have not been properly exposed [153]. However, even lentogenic strains may cause clinical disease, especially in flocks where other pathogens may be circulating [153], and the spread to other flocks [246] or to wild birds [194; 247] has been reported.

Currently used vaccines seem good in most cases to protect against morbidity and mortality induced by the majority of circulating strains [219; 248], but some recent strains may be able to escape the immune response induced by the vaccines available [233; 249]. In addition, vaccination protects the birds against clinical signs but not always against infection and virus shedding albeit vaccines reduce the numbers of birds excreting the virus and the excreted viral titers [209; 250]. These animals may act as asymptomatic carriers of virulent NDV. It has been proposed that strains more closely related phylogenetically would provide better protection [251].

2.7 NDV, a zoonotic virus?

Some authors consider NDV as a zoonotic virus because it has the potential of infecting humans. However, risks are minimal and so far, only one fatal case was attributed to an NDV infection in an immuno-compromised patient [252] and no human-to-human transmission has been reported. Most human cases are work-related infections, linked to mass application of live vaccines, cleaning and disinfecting poultry premises or laboratory acquired infections.

NDV infection in humans manifests primarily as conjunctivitis usually self-limiting and lasting for one or two days [253]. But more generalized infections resulting in chills, headaches and fever, with or without conjunctivitis are also reported less frequently [166].

3. Objectives

The emergence of HPAI H5N1 and its spread beyond the boundaries of the Asian continent lead to an unprecedented epizootic event. Its emergence surprized the West African countries that had never experienced HPAI outbreaks before. The first objective of this study was to monitor poultry for influenza A virus circulation after its introduction in 2006 (**Chapter 1**), mainly in backyard farms and live bird markets which were the most affected parts of the poultry sector in West Africa.

The apparition of HPAI H5N1 in Nigeria without any apparent southward progressive spread was unexpected and therefore migratory birds were suspected to have introduced the viruses in West Africa. Hence, the second objective of this work was to investigate the capacity of migratory birds to carry influenza viruses from Eurasia to Africa (**Chapter 2**). We targeted wild and domestic birds in two regions, one being the major overwintering site for Eurasian migratory birds in Nigeria.

In regions where HPAI viruses are not enzootic, NDV is considered as the main viral threat to the poultry sector, including in West and Central Africa. Although virulent viruses have been isolated in the past, there was no information about the genetic make-up of the strains circulating. There was therefore a need to characterize the circulating viruses (**Chapters 3 and 4**). In a preliminary study (**Chapter 3**), we identified virulent NDV strains that diverged from what had been reported elsewhere. But only partial sequences could be obtained, which was not sufficient to reliably classify them. Therefore, surveillance was maintained and widened to more countries to improve their characterization and have a better understanding of their geographic distribution and genetic diversity (**Chapter 4**).

Similarly to AIV, NDV has a complex ecology and can infect more than 250 bird species, including wild ones. Wild birds are considered as the reservoir of avirulent NDV strains that can sometimes be transmitted to poultry and evolve into virulent strains. Besides some wild birds may carry virulent viruses and could potentially contribute to their spread. Thus we decided to investigate the wild bird reservoir of NDV in West Africa, which had barely been studied before (**Chapter 5**).

The spread of HPAI H5N1 across Europe also urged the set-up of active surveillance of avian influenza in Luxembourg, in which the Institute of Immunology was actively implicated. Active and passive surveillance of NDV has also recently been pushed forward by the European Union. The last objective of this study was to set-up the surveillance for NDV in Luxembourg and to take advantage of the samples collected during AIV surveillance to assess the NDV status in Luxembourg (**Chapter 6**).

Results and Discussion

Chapter 1:

Active surveillance for avian influenza in West Africa: no further evidence of H5N1 circulation after 2008

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Manuscript in preparation

1. Introduction

Before 2006, highly pathogenic avian influenza (HPAI) viruses had rarely been reported in Africa [254; 255]. However, after an outbreak in wild birds at the Qinghai-lake in April 2005 [115], HPAI H5N1 viruses spread to Russia, Europe, the Middle East and eventually reached Africa. The first outbreaks on the African continent occurred in January 2006 in North-West Nigeria [256]. HPAI H5N1 also spread to Niger, Burkina Faso, Côte d'Ivoire, Sudan and Cameroon in 2006 and Ghana, Togo and Benin in 2007. Egypt was also hit in 2006 and, contrary to the other African countries, has not been able to contain HPAI H5N1 virus. Phylogenetic analyses revealed that three sublineages were introduced in Africa and were more closely related to strains from Eastern Europe and the Middle East [66; 257]. During 2006 and 2007, around 300 outbreaks were declared in Nigeria and extensive circulation resulted in the appearance of several reassortant viruses, but only one became widespread [257]. In July 2008, after six months without H5N1 detection, a new sublineage was introduced in Nigeria [127].

HPAI viruses are not only a threat for the poultry industry but also for humans, especially those in direct and frequent contacts with animals, thus justifying on two counts the necessity of surveillance of these viruses. Here we present the results of continued active surveillance in Cameroon and Nigeria between 2006 and 2011. HPAI H5N1 was only detected in 2006 and 2007 in Nigeria, and not after that, suggesting that the virus was properly contained by the control measures undertaken by the local authorities.

2. Material and Methods

2.1 Sample collection

Pools of tracheal and cloacal swabs or cloacal swabs only were collected from domestic species including mainly chickens, but also geese, turkeys, guinea fowls, ducks and pigeons. In Nigeria, samples were collected in 2006 (n= 294), 2007 (n=786), 2008 (n=808), 2009 (n=741) and 2011 (n=534). Three regions were preferentially sampled: South-West where a large portion of commercial farms are localized; North-East, close to the Lake Chad basin and which hosts the biggest wetlands of Nigeria; and North-West representing the northern regions of Nigeria where backyard and free-roaming birds are the main poultry breeding habits. In Cameroon, 300 samples were collected in 2007, 644 in 2009 and 452 in 2011. All

swabs were collected from commercial or backyard farms, live bird markets or free-roaming animals and were placed in virus transport medium [258] upon collection. All samples were shipped to Luxembourg for processing, except for one batch of samples where nucleic acids were purified in Nigeria and shipped.

2.2 RNA extraction, RT-PCR and sequencing

RNA was extracted from 50 µl of virus transport medium using the MagMAX^{TM}-96 AI/ND Viral RNA Isolation Kit (Life Technologies, Merelbeke, Belgium) with the KingFisher 96 (Thermo Fisher, Waltham, Massachusetts, USA) or from 140 µl of medium using QIAamp Viral RNA Mini Kit (Qiagen, Venlo, The Netherlands). Influenza A matrix gene, specific H5 and N1 detection real-time RT-PCRs were performed as previously described [123]. Matrix positive samples were also screened with specific H7 real-time RT-PCR [259]. Hemagglutinin (HA), neuraminidase (NA), matrix (MP), nucleoprotein (NP) and non-structural (NS) protein genes from the samples with the lowest Ct values in the matrix detection RT-PCR were amplified as several overlapping fragments (primer sequences and details available upon request). Due to the restricted amount of material available, sequencing of the polymerase complex genes was not attempted. PCR products were purified using the JetQuick PCR Purification Spin kit (Genomed, Loehne, Germany) or QIAquick Gel Extraction Kit (Qiagen) when multiple bands were visible. Sequencing was performed using PCR primers as sequencing primers. PCR products were sequenced in the both directions using the Big Dye Terminator v.3.1 cycle sequencing kit (Life Technologies) and ABI 3130 Avant capillary sequencer (Applied Biosystems).

2.3 Phylogenetic analyses

Sequences were assembled, analyzed and aligned using SeqScape v2.5 (Applied Biosystems) and BioEdit [260]. Pair-wise genetic distances were calculated with the Neighbour-Joining, Kimura-2 parameter model (MEGA v5.03, [261]). The sequences generated in this study were compared to all H5N1 strains from Asia and Europe available on GenBank and to all African strains available on EpiFlu^{TM} Database (http://platform.gisaid.org). Phylogenetic analyses were performed with Neighbour-Joining, Kimura-2 parameter model and 1000 bootstrap replicates. Based on these analyses, representative strains were selected and are shown in Figure 9 for HA gene and Figure 10 for NA gene. The official nomenclature for

HPAI H5N1 was used for the major defined clades [262] and denomination of A and C was used for the two predominant Nigerian sublineages [124].

3. Results

3.1 Sampling

In Nigeria, influenza A positive samples were found in 2006 (143/294) and 2007 (30/786). There were found in 14 live bird markets, 20 farms and 6 unspecified locations. No matrix positive samples were detected in 2008, 2009 and 2011 in Nigeria nor in 2007, 2009 and 2011 in Cameroon. Only 103/173 matrix positive samples were confirmed to be H5 positives, but none were H7 positive. In most cases (35 locations), at least one of the samples was H5 positive. The Ct values of the matrix real-time RT-PCR of the samples collected in the five locations where no H5 cases could be confirmed ranged from 29 to 34.

3.2 Phylogenetic analyses

Partial or complete sequences of HA, NA, MP, NP and NS genes were obtained for 47 samples and were compared to available sequences of H5N1 viruses from Asia, Europe and Africa. Phylogenetic analyses based on HA gene revealed that all strains clustered in sublineage A except 5 strains from a single farm in Oyo state in 2007 that belonged to the $AC_{HA/NS}$ reassortant sublineage (Figure 9). Phylogenetic analyses on NA (Figure 10), NP and NS genes revealed similar results (data not shown). The distinction between sublineage A and $AC_{HA/NS}$ was not visible based on the analyses of the matrix gene due to low number of mutations between the two groups of viruses for this gene. When comparing clustering of strains for each gene, neither reassortment (based on 5 genes only) between sublineages nor within sublineage reassortment could be identified.

Samples collected in the same market or in the same farm were always more closely related to each other than to strains found in other locations. Kimura distances of samples from the same farm ranged from 0 to 0.5% for each gene. However, phylogenetic analyses of samples A/chicken/Nigeria/152/2006, A/chicken/Nigeria/158/2006, A/chicken/Nigeria/159/2006 and A/chicken/Nigeria/164/2006 which were collected in a single market in Oyo state showed that these strains were not most closely related to each other, revealing that a higher heterogeneity was found in this market (Figure 9 and Figure 10).

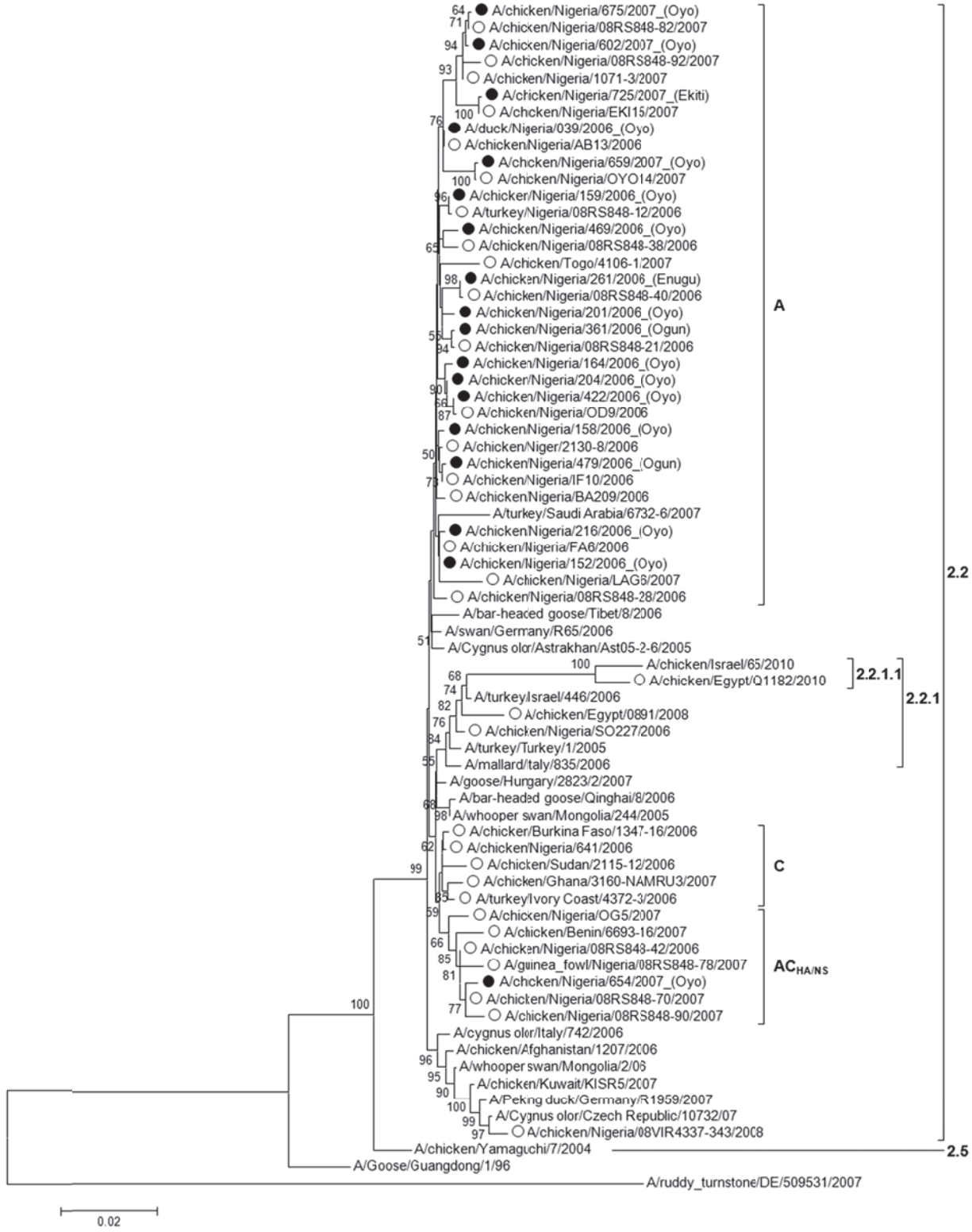

Figure 9. Phylogenetic analyses of partial (nt 55 to 1674) of HA gene. Only one strain per sampling location is shown, except for four strains from a single live bird market. African strains are marked with an open circle and strains sequenced in this study are marked with a close dot. Only bootstrap values higher than 50% are shown.

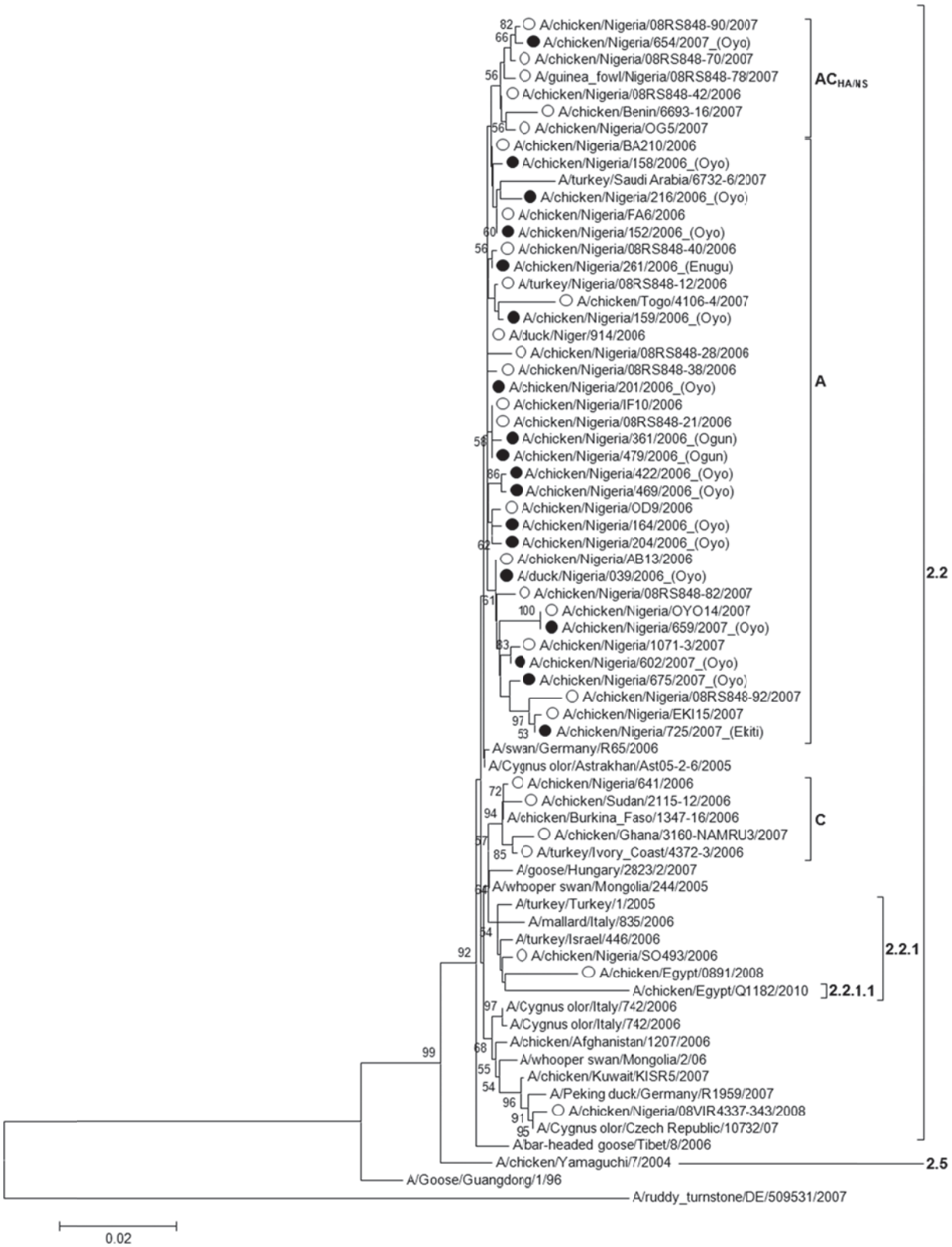

Figure 10. Phylogenetic analyses of partial (nt 55 to 1368) of NA gene. Only one strain per sampling location is shown, except for four strains from a single live bird market. African strains are marked with an open circle and strains sequenced in this study are marked with a close dot. Only bootstrap values higher than 50% are shown.

3.3 Molecular characterization

Examination of the deduced amino acid sequences showed that all the strains shared 226Q and 228G on the HA (H3 numbering), indicating higher affinity for binding to sialic acid α2,3. No marker of drug resistance against adamantanes on the matrix gene nor against neuraminidase inhibitors were present.

The HA cleavage site sequences were identical to the one exhibited by the majority of Nigerian strains. Only two strains A/chicken/Nigeria/467/2006 and A/chicken/Nigeria/469/2006 from the same farm had a R330K mutation, but variations in the cleavage site sequence of Nigerian strains were already observed on eight other occasions (Table 5).

Table 5. HA cleavage site sequence variability among Nigerian HPAI H5N1 strains

Cleavage site	Strain
PQGERRRKKR*GLF	Majority of Nigerian strains
PQG**K**RRRKKR*GLF	A/chicken/Nigeria/08RS848-76/2007
	A/chicken/Nigeria/08RS848-83/2007
PQ**R**ERRRKKR*GLF	A/chicken/Nigeria/08VIR4337-378/2006
	A/chicken/Nigeria/08VIR4337-380/2006
	A/chicken/Nigeria/VRD44/2006
	A/chicken/Nigeria/VRD49/2006
PQGE**K**RRKKR*GLF	A/chicken/Nigeria/08RS848-90/2007
PQGERRR**R**KR*GLF	A/chicken/Nigeria/1071-9/2007
PQGER**K**RKKR*GLF	A/chicken/Nigeria/467/2006
	A/chicken/Nigeria/469/2006

4. Discussion

In our study, influenza A viruses were only detected in 2006 and 2007. Not all matrix positive samples could be confirmed to be of the H5 subtype, which could partially be explained by a slight difference in sensitivity of the two RT-PCRs. However in most cases, H5 was confirmed in at least one other sample collected at the same location. Other groups carrying surveillance studies in Nigeria during the same period never reported the detection of other subtypes despite the high total number of samples analyzed [122; 131; 257]. Nevertheless, we cannot exclude that other non-H5, non-H7 strains were present in Nigeria at the time of the surveillance.

Phylogenetic analyses of the strains sequenced confirmed the circulation in 2006 of sublineage A and of the co-circulation in 2007 of sublineage A and $AC_{HA/NS}$ reassortant, which likely emerged already in 2006 [257]. Although $AC_{HA/NS}$ reassortants seemed to have

become predominant and constituted the majority of the isolates reported throughout Nigeria during 2007 [257], we confirmed that sublineage A strains were still circulating in South-Western states at least until September 2007.

Focus on the five shortest genes was decided based on the majority of reassortants only showing exchanges of HA, NS or NP genes [129; 130; 257]. Only one other type of reassortment, referred to as $AC_{HA/NS/NP/PB1}$, involved the polymerase genes [128]. No additional reassortant was found in our study but it would have been interesting to obtain sequences of the remaining three genes. The small amount of material available and the low concentrations of nucleic acids limited the possibility to amplify easily the complete genome of all strains.

Despite still much debate concerning the introduction route of HPAI H5N1 virus in Africa, there is no doubt about the role played by human activities, especially movement and trade of poultry, in the spreading of the virus within Nigeria. Recent studies identified biosecurity measures, proximity to other farms, allowing entry of external people in the farm and purchase of live poultry products as risk factors associated with HPAI H5N1 virus infection in Nigerian farms [263; 264]. Joannis *et al.* suggested that the peaks of influenza detected cases in January/February in 2006 and 2007 was likely due to the increasing movements of birds around Christmas and New Year festive periods [265]. Our finding of various strains of sublineage A in one live bird market which suggested that the strains found may have had separate origins, together with the geographic scattering of highly similar strains [257], illustrates that trade, and particularly in live bird markets, likely played an important part in spreading HPAI H5N1 virus.

In our study, no AIV positive samples were found after 2007. Although we cannot exclude partial RNA degradation during storage and shipment, other RNA viruses such as NDV and IBV were detected in the same batch of samples suggesting that sample degradation cannot entirely account for the absence of AIV detection. Likewise, surveillance studies performed in Côte d'Ivoire, Benin and Togo between November 2008 and December 2010 also failed to detect AIV in live-bird markets and backyard farms [266]. Three years of surveillance in live bird markets and commercial farms in Bangui, Central African Republic, did not detect H5 positive samples (GF Komoyo, pers. comm.). Also no avian influenza cases in sub-Saharan Africa, besides South Africa, were notified to the OIE after 2008. Taken together, this would suggest that HPAI H5N1 was successfully controlled in the region.

However, other influenza strains are likely circulating in domestic poultry in sub-Saharan Africa. Among backyard poultry sampled in villages near the wetlands of the inner delta of the Niger in Mali, 3.6% were positive by RT-PCR and 13.7% of the poultry had anti-AIV antibodies [267]. Recently, influenza A virus was detected in 0.8% of poultry in live bird markets in Kenya [268] and South Africa is again experiencing HPAI H5N2 cases in commercial ostrich farms since beginning of 2011. In addition, H9N2 viruses were reported in wild birds and poultry in Tunisia since 2010 [269] and Egypt since 2011 [270], and Egypt was still not able to control HPAI H5N1 since its introduction in 2006. The burst of studies screening wild birds in Africa for the presence influenza A in the aftermath of HPAI H5N1 virus outbreaks also identified several subtypes in Zambia [271], Nigeria [272; 273], in Mali, Ethiopia and Senegal [274] and Egypt [275]. These studies also revealed that influenza viruses can be maintained in the wild bird reservoir in Africa [273]. Therefore, the apparent eradication of HPAI H5N1 virus in West and Central Africa should not lead to decreased virus surveillance.

5. Acknowledgments

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Chapter 2:

Reassortant low pathogenic avian influenza H5N2 viruses in African wild birds

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1. Introduction

Wild birds, in particular of the Charadriiformes and Anseriformes orders, are considered natural asymptomatic reservoirs of low pathogenic avian influenza (LPAI) virus [9; 276] and the source of influenza viruses in other species including poultry [9]. All subtypes (H1-16, N1-9) of avian influenza have been found in wild birds [277; 278]. Migration after the breeding season along distinct flyways contributes to long-distance dissemination of influenza viruses [75] and the aquatic environment of waterfowl supports efficient short-range virus transmission of LPAI viruses by the faecal-oral route [9; 76]. High density of mixed bird populations at stopovers and migration sites also promote intra- and interspecies virus transmission [77].

Wetlands in Africa are preferred non-breeding sites for many Eurasian migratory waterbirds. Although Eurasian species can mix in many important bird areas with resident African birds or intra-African migrants [279; 280], only a few avian influenza viruses (AIV) have been reported from Africa before 2006 [57; 58; 254; 255; 281-286]. The first seroprevalence study in sub-Saharan Africa conducted in commercial poultry in Nigeria between 1999 and 2004 did not detect antibodies to influenza viruses [120]. In a first wild bird surveillance effort, a wide variety of LPAI viruses were identified in migratory waterbirds [274]. Interest in AIV in wild birds in Africa further increased in 2006 when highly pathogenic avian influenza (HPAI) H5N1 virus was first identified in Africa in Nigerian poultry farms [121]. After the independent introduction of three sublineages of clade 2.2 viruses into poultry, HPAI H5N1 viruses have undergone multiple reassortments and reassorted viruses have largely replaced the initial sublineages [129]. The route of introduction of H5N1 is unknown and both migratory birds and poultry trade may be incriminated [123; 124; 127]. Although efforts to isolate influenza viruses from wild birds in Africa intensified, few met with success [66; 286]. Vultures have probably been infected by scavenging on H5N1 infected poultry [66] but the infection route of the Common Teal (*Anas crecca*) from Egypt is not known [286].

In this study, we investigated the presence of avian influenza in wild and domestic birds in two different locations in Nigeria in 2008. The Dagona Wildlife Sanctuary is a protected area within the Hadejia-Nguru Wetlands, located in Northeastern Nigeria, and is part of the Chad Basin National Park. Large numbers of migratory birds mix in the Hadejia-Nguru Wetlands with local wild birds. The Hadejia-Nguru Wetlands are also the most important bird area in the region from where first HPAI H5N1 outbreak in Africa was reported. The Amurum

Forest Reserve is also a protected area located 15 km northeast of Jos (Plateau State), very close to the urban community [279]. We did not find any HPAI H5N1 virus but LPAI H5N2 strains that contained genes from the Eurasian gene pool and a NS gene most closely related to other African viruses. Interestingly the HA genes were part of a cluster that contained also HA genes from HPAI H5N2 viruses found in the same bird species and in the same region one year earlier.

2. Material and Methods

2.1 Wild bird surveillance

Wild birds were captured with mist nets in the Dagona Wildlife Sanctuary in Northeastern Nigeria between March 28th and April 22nd, 2008. Oropharyngeal and cloacal swabs as well as fresh faecal samples were collected after ensuring the species of origin. Domestic poultry in the villages around the wetlands were also sampled. All samples were collected in triplicates with cotton swabs, stored in virus transport medium (PBS pH 7.0 with 2000 U/ml Penicillinl, 200 mg/ml streptomycin, 2000 U/ml polymyxin B, 250 mg/ml gentamicin, 60 mg/ml ofloxacin, 200 mg/ml sulfamethoxazole and 2.5 mg/ml amphotericin B) and placed directly in the field in liquid nitrogen. In addition, between December 4th, 2007 and March 5th, 2008, swabs were collected from wild birds in the Amurum Forest Reserve (Plateau State) and from backyard poultry in five villages around Jos.

2.2 RNA Extraction, RT-PCR and sequencing

RNA was extracted from 50 µl of virus transport medium using the MagMAX^{TM}-96 AI/ND Viral RNA Isolation Kit (Ambion) with the KingFisher 96 (Thermo Fisher). Influenza A positive specimens were detected by using a real-time RT-PCR assay targeting the matrix gene and previously published gene-specific primers [287] and probe [123]. RT-PCR reactions were carried out using the following cycling conditions: reverse transcription for 30 min at 50°C, denaturation at 95°C for 15 min, 40 cycles of amplification at 95°C for 10 s, 60°C for 20 s. Amplification were performed with the QIAGEN OneStep RT-PCR Kit using 2 µl of RNA in a final volume of 25 µl. Matrix positive samples were tested for H5 [123], H7 (http://www.defra.gov.uk/vla/science/docs/sci_ai_vi536.pdf) and N1 [288] genotypes. The 8 genes were then amplified with several PCRs targeting overlapping fragments (primer

sequences and details available upon request). PCR products were purified using the JetQuick PCR Purification Spin kit (Genomed). Sequencing was performed as previously described [289] using PCR primers as sequencing primers.

2.3 Molecular and phylogenetic analyses

Sequence assembly and analyses were performed using SeqScape v2.5 (Applied Biosystems) and Bioedit [260]. The nucleotide sequences are available in the DDBJ/EMBL/GenBank databases under the accession numbers FR771823 to FR771846. Potential N-linked glycosylation sites were predicted in HA and NA by the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Genetic distances were calculated with MEGA4 [290] with the Kimura 2-parameters model. For each gene, phylogenetic relationships were inferred by comparing the LPAI H5N2 strains with all avian influenza strains downloaded on NCBI Influenza Virus Resource (15.12.2009) after removal of short sequences and sequences with insertions or deletions resulting on frame shifts. Datasets were aligned using ClustalW [291]. Coding regions were used for phylogenetic analyses and only the first open reading frames were used for M and NS genes. Trees were calculated using MEGA4 [290] with Neighbour-Joining method, using the Kimura 2-parameters model and 1000 bootstrap replicates. Representative strains were selected for each gene based on these preliminary analyses.

Tree topologies, substitution rates and TMRCAs were estimated by a Bayesian Markov Chain Monte Carlo (MCMC) method [292] implemented in BEAST v1.5.3 software [293]. Depending on the available details on isolation dates, the exact isolation dates, the mid-month dates (15^{th}), the mid-interval dates or the mid-year dates were used as calibration points. For each dataset, different substitution models with 2 codon partitions to allow independent estimates for the third codon position, two uncorrelated relaxed clock models (lognormal and exponential distributions; [294] and two coalescent models (constant population size and Bayesian skyline; [295] were compared visually in Tracer 1.5.3 [296] and statistically using a Bayes Factors (the ratio of the marginal likelihoods of two models) test [297; 298] as implemented in Tracer v1.5.3 in order to identify the model that fitted the data best. Evidence against the null model, which is the model with the lowest marginal likelihood, was assessed by the method proposed by Kass and Raftery [297]. When there was no evidence against the null model or when this evidence was weak, the simplest model was kept to avoid unnecessary over-parameterization. Analyses revealed that the general time reversible (GTR)

substitution model assuming an uncorrelated exponential relaxed clock and a constant population size was the model that best fitted the data for all genes. For M, NP and NS, the GTR model was further simplified to avoid over-parameterization (Table 6). Two to three runs of 50 to 100 million generations of the MCMC chain were performed and sampled to produce 10 000 trees each. Convergence of the runs was confirmed in Tracer 1.5.3. The results of multiple runs were combined using LogCombiner v1.5.3 [293] with a burn-in of 10% to 25%, summarized into the Maximum Clade Credibility tree using TreeAnnotator v1.5.3 [293] and visualized in FigTree v1.3.1 [299].

Table 6. Estimated TMRCAs of the Nigerian LPAI H5N2 cluster, nucleotide substitution model and sequence length used in the Bayesian analyses.

Gene	Mean TMRCA (95% HPD)	Nucleotide substitution model	Sequence length
PB2	March 2007 (Jun 05 – Mar 08)	GTR + I + Γ*	1593 nt
PB1	June 2007 (Jun 06 – Mar 08)	GTR + I + Γ	1692 nt
PA	March 2007 (Jul 05- Feb 08)	GTR + I + Γ	2151 nt
HA	October 2007 (Feb 07 – Mar 08)	GTR + I + Γ	1710 nt
NP	January 2007 (Jun 05 – Feb 08)	GTR + I + Γ with simplifications : CP3.cg=CP3.gt, CP1+2.at=CP1+2.gt=CP1+2.cg	855 nt
NA	August 2007 (Oct 06 – Mar 08)	GTR + I + Γ	1377 nt
M	December 2006 (Jun 05 – Jan 08)	GTR + I + Γ with simplifications : CP3.cg=CP3.at, CP1+2.at= CP1+2.cg	759 nt
NS	October 2004 (Jan 02 – Feb 07)	GTR + Γ with simplifications : CP3.cg=CP3.gt	690 nt

*General time-reversible (GTR) substitution model with a gamma (Γ) and invariant (I) site heterogeneity model.

3. Results

3.1 Avian influenza prevalence is low

A total of 1024 samples were collected in the Dagona Wildlife Sanctuary from 44 wild bird species, including waterfowl, waders and passerines, and from 373 domestic poultry such as chickens, ducks and geese (Table 7). Around Jos, cloacal samples were collected from 374 domestic birds (362 chickens, 5 ducks and 7 turkeys). In addition 154 wild birds corresponding to 52 species, mainly passerines, were sampled in the Amurum Forest Reserve (Table 7). Only three fresh faecal samples collected from Spur-winged Geese *Plectropterus gambensis* in a single location in the Dagona Wildlife Sanctuary (12°44.676 N, 10°40.001 E)

were Influenza A positive. They were collected on March 31st (A/spur-winged goose/Nigeria/2/2008) and on April 3rd (A/spur-winged goose/Nigeria/210/2008, A/spur-winged goose/Nigeria/226/2008). Thus, in our study, the prevalence of AIV in the Dagona Wildlife Sanctuary was 0.3% (3/1024). All samples collected in Plateau State were Influenza A negative.

3.2 Molecular analyses show no marker of virulence

Genotype-specific PCRs were positive for H5 and N2 for the three viruses and all genes were sequenced using previously published or newly designed primers. Analyses of the HA gene showed that the predicted amino acid (a.a.) sequence of the cleavage site of the three viruses corresponded to a low pathogenic pathotype (PQRETR*GLF). They had a glutamine at position 226 and a glycine at position 228 (H3 numbering) indicating a higher binding affinity for sialic acid α2,3, characteristic of avian cell-surface receptors [28; 300]. No stalk deletion in the NA gene, nor additional predicted glycosylation sites, both proposed to be associated with poultry adaptation, were detected [79; 301]. No genetic marker associated with increased virulence in mammals (PB2 627E, 701D, 714S; PB1 678S; PA 615K; NP 319N; [43] or drug resistance (M2 26L, 27V, 30A, 31S, 34G [151]; NA 119E [302]; 274H [303]; 292R [302]; 294N [302; 304]) were found.

Table 7. List of sampled domestic and wild bird species (Order and Family) in Dagona Wildlife Sanctuary and in the Amurum Forest Reserve and villages around Jos, Nigeria in 2008. For each Family, the number of samples from domestic birds is mentioned in brackets.

Order	Family	Dagona Wildlife Sanctuary		Amurum Forest Reserve and villages around Jos	
		No. species/ Family	No. samples/ Family	No. species/ Family	No. samples/ Family
Accipitiformes	Accipitridae	2	4		
Anseriformes	Anatidae	4	262 (133)	1	5 (5)
Charadriiformes	Jacanidae	2	32		
	Scolopacidae	2	18		
Ciconiiformes	Ardeidae	1	3		
Coliiformes	Coliidae			1	9
Columbiformes	Columbidae	6	162	2	4
Coraciformes	Alcedinidae	2	20	1	1
	Coraciidae	1	1		
Cuculiformes	Cuculidae	1	1	1	1
Galliformes	Meleagrididae			1	7 (7)
	Phasianidae	1	240 (240)	1	362 (362)
Gruiformes	Rallidae	1	1		
Piciformes	Indicatoridae	1	1	1	2
	Lybiidae			4	16
Passeriformes	Alaudidae			1	1
	Dicruridae	1	1		
	Emberizidae			1	1
	Estrildidae	5	14	6	38
	Fringillidae	1	37		
	Hirundinidae	3	9		
	Malaconotidae			3	7
	Monarchidae			1	2
	Motacillidae	2	14	1	1
	Muscicapidae			1	3
	Nectariniidae			3	8
	Passeridae	3	56	2	2
	Platysteiridae			1	1
	Ploceidae	5	123	4	12
	Pycnonotidae			1	3
	Sylviidae	1	4	13	32
	Turdidae			2	5
	Viduidae	2	21	1	1
	Zosteropidae			1	4
Total		**47**	**1024**	**55**	**528**

3.3 Phylogenetic analyses of the 8 genes reveal a separate origin for the NS gene

To explore the evolutionary origin and the time of the introduction of LPAI H5N2 in Nigeria, tree topologies were assessed and times to the most recent common ancestor (TMRCAs) were estimated using BEAST v1.5.3. For each gene, the three LPAI H5N2 viruses were genetically highly similar (Kimura distance from 0 to 0.9%) and always clustered together (100 % posterior probabilities for all genes), suggesting that the three strains recently evolved from a common ancestor and resulted from a single introduction event in the Nigerian wetlands. All genes belonged to the Eurasian and not the American lineage, but none of them were related to HPAI H5N1 strains from Nigeria, from other parts of Africa or from Eurasia.

Phylogenetic analyses of the hemagglutinin gene revealed that the LPAI H5N2 viruses were most closely related to a European H5N3 isolate (A/duck/Tulcea/RO-AI-LPAI/2009) recently found in a sentinel duck in Romania (Figure 11). The neuraminidase genes of the LPAI H5N2 viruses were most closely related to a H2N2 strain from the Netherlands (A/mallard/Netherlands/14/2007) (Figure 12). The LPAI H5N2 viruses formed a sister clade to strains recently isolated from wild birds in Europe (HA and NA) and Asia (NA). Interestingly they also formed a sister branch with highly pathogenic H5N2 strains isolated one year earlier in the same location (10 km away) from the same wild bird species (HA and NA). In the same HA cluster, closely related HPAI and LPAI H5N2 viruses both from South African ostriches (A/ostrich/South Africa/AI1160/2006, LPAI; A/ostrich/South Africa/AI1091/2006, HPAI) formed another sister branch to the Nigerian LPAI H5N2 viruses. However all African isolates did not share a direct common ancestor, suggesting that they did not directly evolve from each other. The TMRCAs of the LPAI H5N2 HA and NA genes ranged from February 2007 to March 2008 (95% highest posterior density interval, HPD) and October 2006 to March 2008 (95% HPD) respectively.

The PA gene of the LPAI H5N2 viruses formed a sister clade to PA genes from a goose and wild and captive ducks from France, the Netherlands and Sweden (Figure 14). The TMRCA of the LPAI H5N2 cluster was estimated between July 2005 and February 2008 (95% HPD). The PB1 gene showed that the LPAI H5N2 viruses formed a sister clade with two wild bird viruses from the Netherlands (A/turnstone/Netherlands/1/2007, H3N8; A/mallard/Netherlands/17/2007, H11N8) and a virus from one domestic goose from Czech Republic (A/goose/Czech Republic/1848/2009, H7N9) (Figure 15). The TMRCA of the LPAI H5N2 cluster was estimated between June 2006 and March 2008 (95% HPD). The PB2

genes of the 3 H5N2 strains were most closely related to A/quail/Italy/4610/2003 (H7N2) and the TMRCA of the LPAI H5N2 cluster ranged from June 2005 to March 2008 (95% HPD) (Figure 16).

The matrix gene sequences were more closely related to a H9N2 matrix gene from the Netherlands (A/Bewick's swan/Netherlands/5/2007) and the TMRCA ranged from June 2005 to January 2008 (95% HPD) (Figure 17). The 3 NP genes clustered with genes from four ducks, one swan and one turnstone viruses from Italy, the Netherlands, Hungary, Germany and Sweden, and the TMRCA of the cluster formed by these 3 genes ranged from June 2005 to February 2008 (95% HPD) (Figure 18).

The NS sequences clustered in the allele B and formed a sister group to three South African NS sequences (A/ostrich/South Africa/AI1091/2006, H5N2; A/duck/South Africa/1108/2004, H3N8; A/duck/South Africa/1233A/2004, H4N8) and to H3N6 gene from Zambia (A/pelican/Zambia/01/2006) (Figure 13). Larger 95% HPD values of the TMRCA of the Nigerian LPAI H5N2 cluster were observed (January 2002 to February 2007), which may indicate an older origin of that gene, although we cannot exclude that our dataset for the NS segment does not contain sufficient phylogenetic signal to provide precise estimates. Also the exclusion of the distantly related NS gene of A/duck/NZL/160/1976 (H1N3) from the phylogenetic analysis had little influence on the TMRCA. The close relationship suggests that all seven viruses shared a recent common ancestor that was introduced in Africa (TMRCA of the African NS cluster, node II in Figure 13: November 1997 to May 2003).

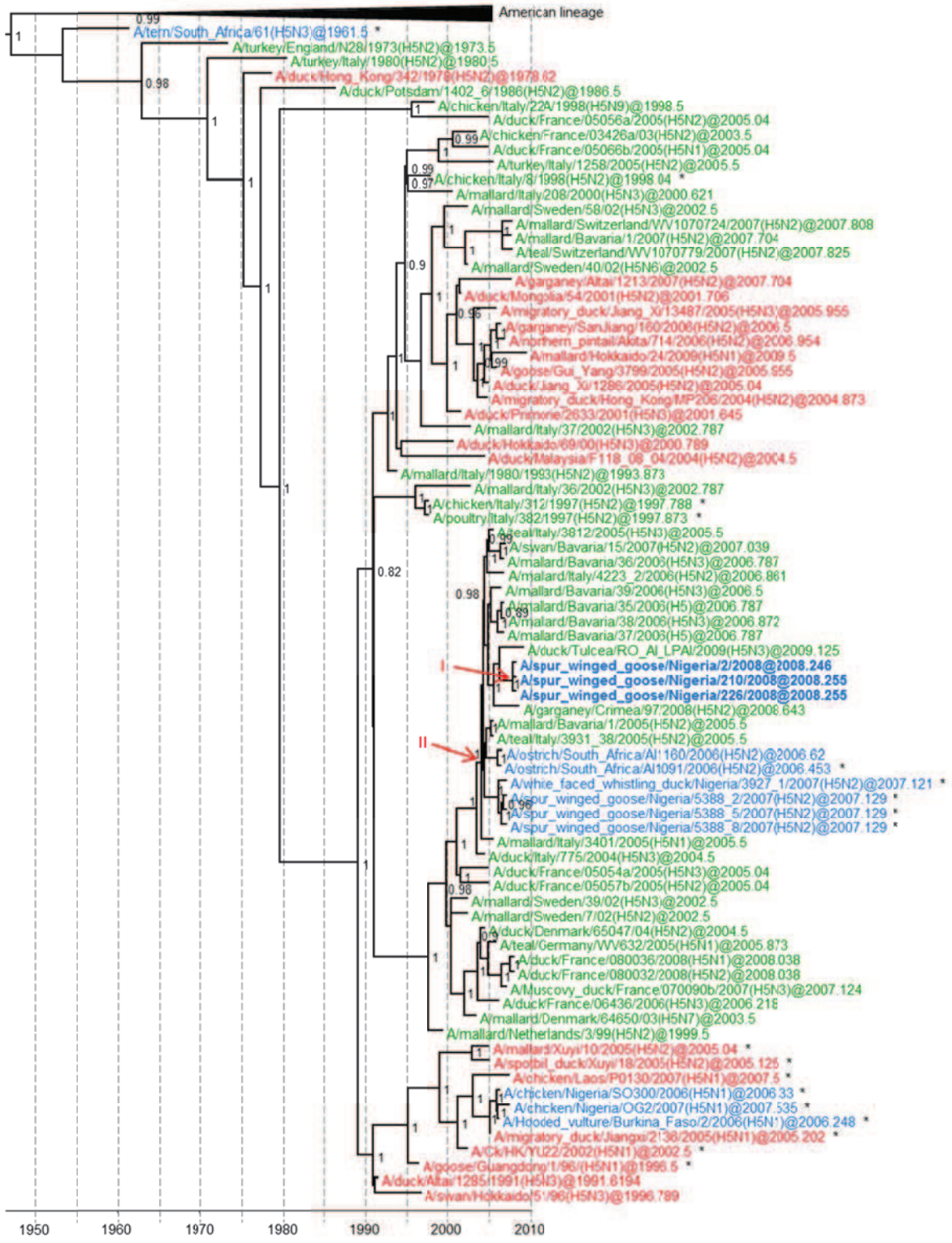

Figure 11. Dated phylogeny of H5 genes. The horizontal axis represents calendar years and nodes correspond to mean TMRCAs. Posterior probability values higher than 0.75 are shown. Representative strains from Africa (blue), Europe (green) and Asia (red) are included. Virus strains characterized in this study are presented in bold. Black stars indicate HPAI strains. Node I represents the mean TMRCAs of the LPAI H5N2 cluster, and node II corresponds to the most recent common ancestor of the Nigerian LPAI and HPAI supported by a high posterior probability value.

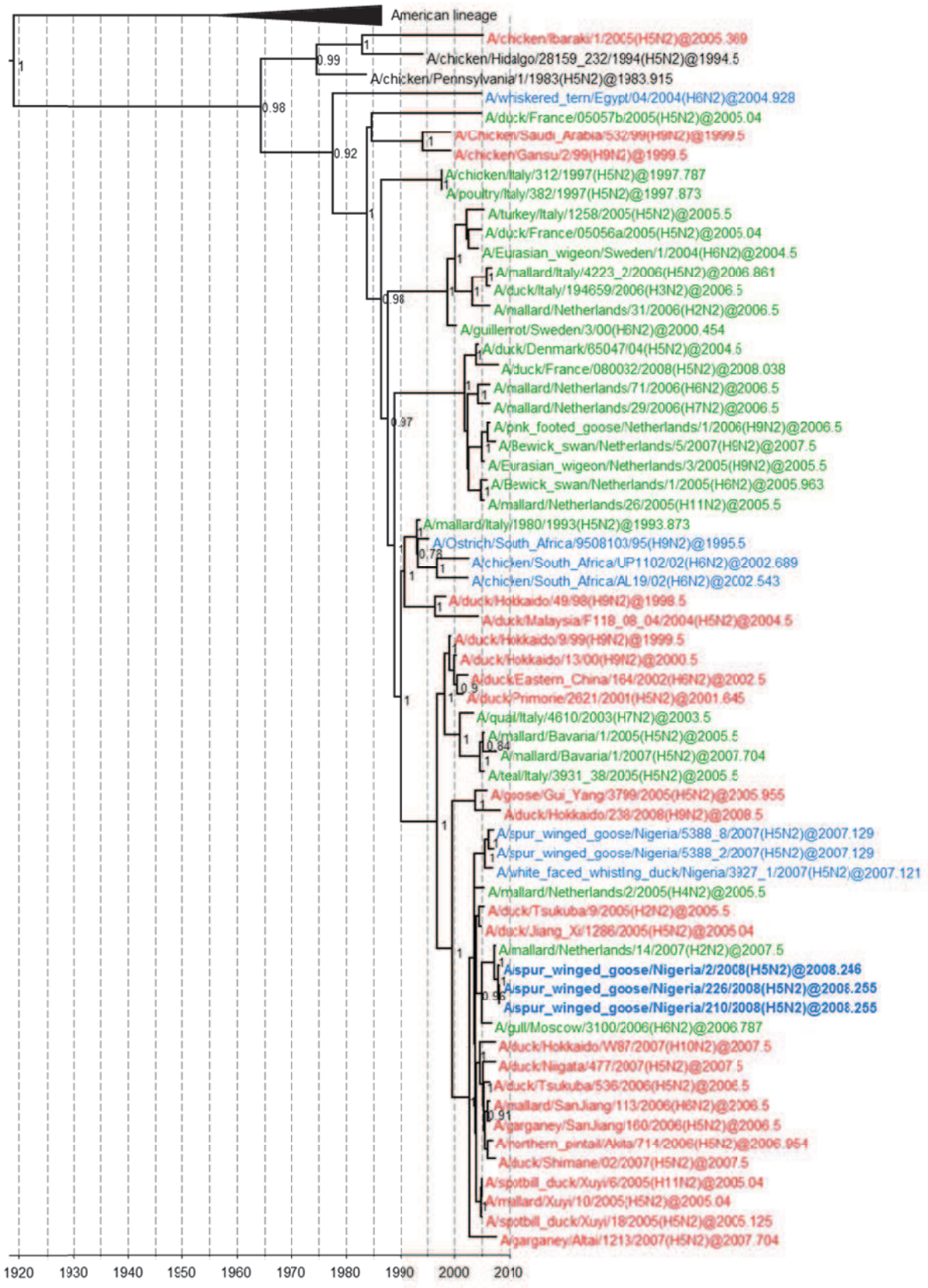

Figure 12. Dated phylogeny of NA genes. The horizontal axis represents calendar years and nodes correspond to mean TMRCAs. Posterior probability values higher than 0.75 are shown. Representative strains from Africa (blue), Europe (green) and Asia (red) are included. Virus strains characterized in this study are presented in bold.

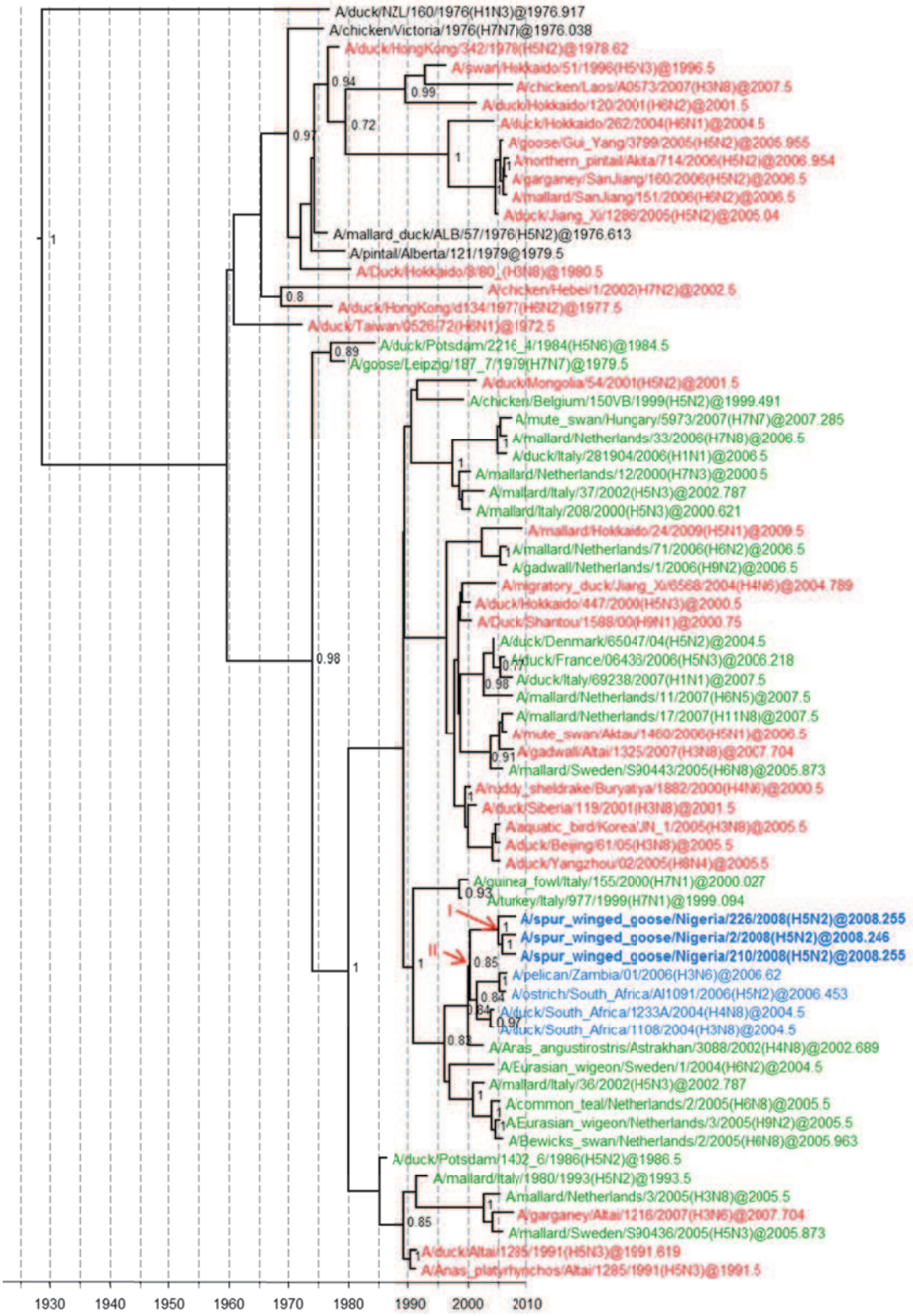

Figure 13. Dated phylogeny of NS genes clustering in allele B. The horizontal axis represents calendar years and nodes correspond to mean TMRCAs. Posterior probability values higher than 0.75 are shown. Representative strains from Africa (blue), Europe (green) and Asia (red) are included. Virus strains characterized in this study are presented in bold. Node I represents the mean TMRCAs of the LPAI H5N2 cluster, and node II corresponds to the most recent common ancestor of the African NS genes, including the Nigerian LPAI H5N2.

Figure 14. Dated phylogeny of PA genes. The horizontal axis represents calendar years and nodes correspond to mean TMRCAs. Posterior probability values higher than 0.75 are shown. Representative strains from Africa (blue), Europe (green) and Asia (red) are included. Virus strains characterized in this study are presented in bold.

Figure 15. Dated phylogeny of PB1 genes. The horizontal axis represents calendar years and nodes correspond to mean TMRCAs. Posterior probability values higher than 0.75 are shown. Representative strains from Africa (blue), Europe (green) and Asia (red) are included. Virus strains characterized in this study are presented in bold.

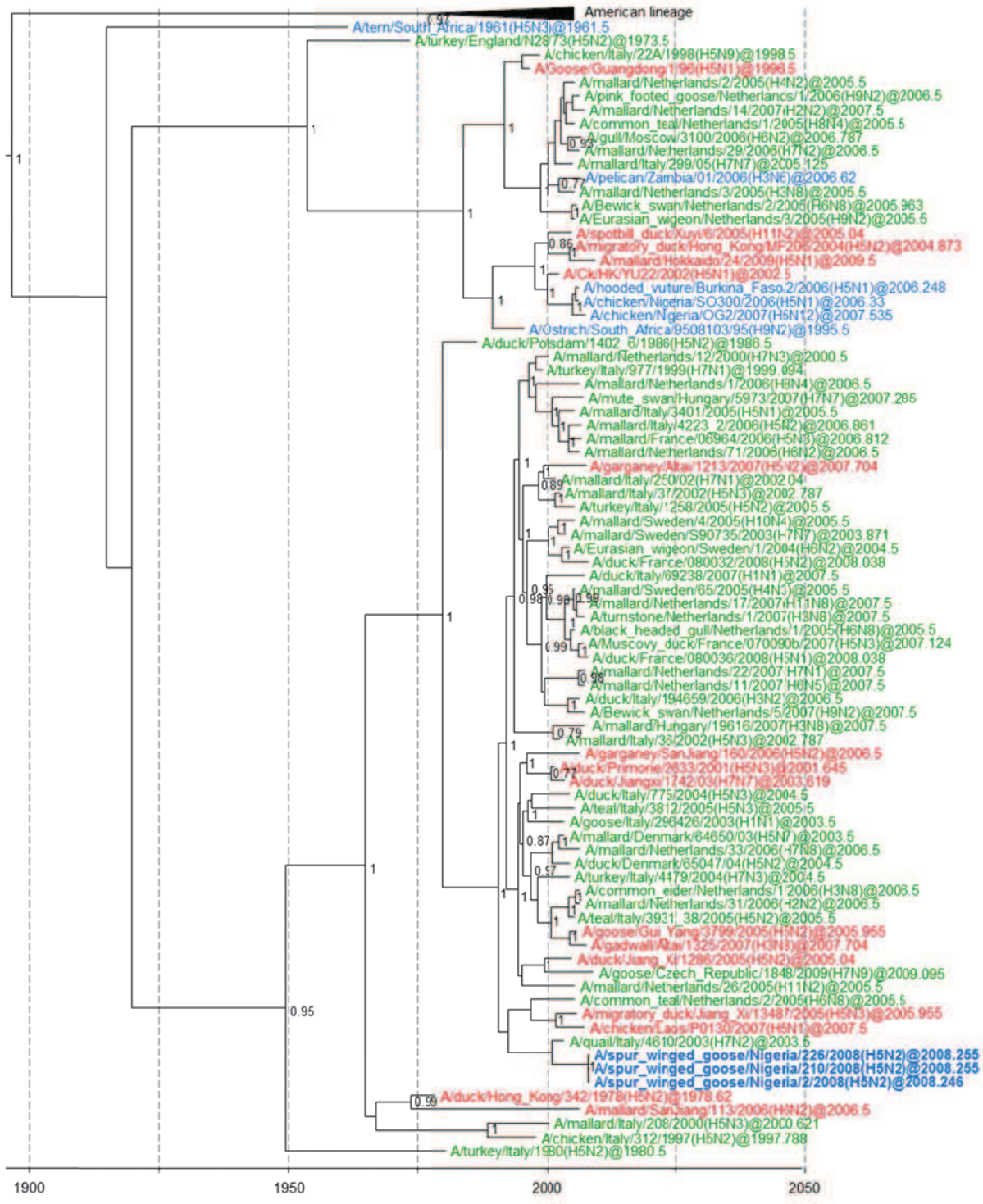

Figure 16. Dated phylogeny of PB2 genes. The horizontal axis represents calendar years and nodes correspond to mean TMRCAs. Posterior probability values higher than 0.75 are shown. Representative strains from Africa (blue), Europe (green) and Asia (red) are included. Virus strains characterized in this study are presented in bold.

Figure 17. Dated phylogeny of M genes. The horizontal axis represents calendar years and nodes correspond to mean TMRCAs. Posterior probability values higher than 0.75 are shown. Representative strains from Africa (blue), Europe (green) and Asia (red) are included. Virus strains characterized in this study are presented in bold.

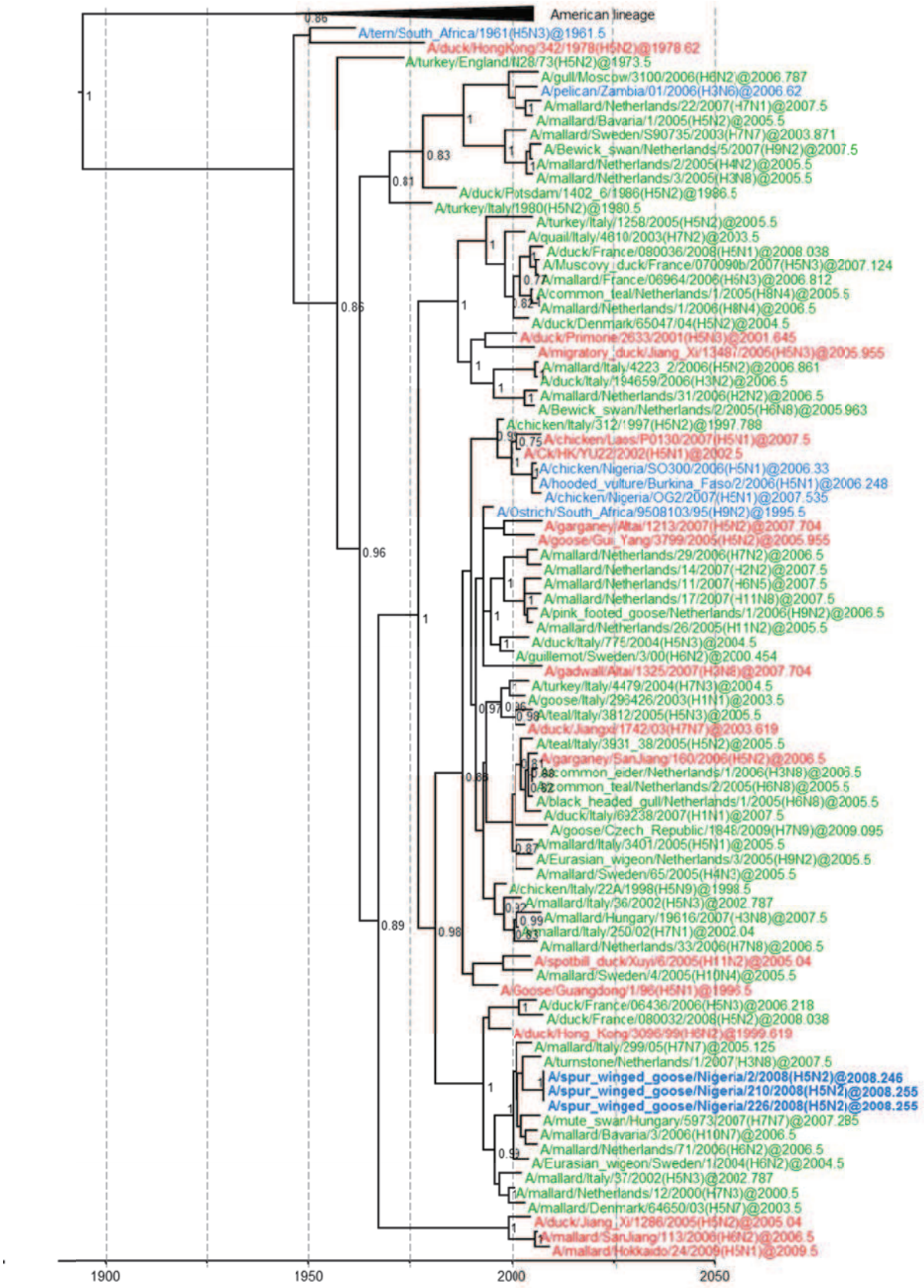

Figure 18. Dated phylogeny of NP genes. The horizontal axis represents calendar years and nodes correspond to mean TMRCAs. Posterior probability values higher than 0.75 are shown. Representative strains from Africa (blue), Europe (green) and Asia (red) are included. Virus strains characterized in this study are presented in bold.

4. Discussion

Although avian influenza has been extensively studied for decades in wild birds from the Northern Hemisphere, with few exceptions [57; 58; 254; 255; 281-285], AIV has received attention in Africa only after the introduction of HPAI H5N1 viruses. In our survey, three out of 1024 (0.3%) samples collected in the Dagona Wildlife Sanctuary, a part of the Hadejia-Nguru Wetlands, in Northeastern Nigeria were positive for Influenza A. This corresponds to a prevalence of 1% (3/312) in birds from the Charadriiformes and Anseriformes orders which are considered the natural reservoir of AIV [9]. In Spur-winged Geese we found an infection rate of 2.4% (3/123) compared to 8.2% (8/97) in the earlier study [272]. The three viruses found were low pathogenic H5N2 viruses most closely related to each other over the full genome. A 10 times higher prevalence (3.9%) was found in waterbirds in the Hadejia-Nguru Wetlands in the previous year [272]. This is similar to the overall prevalence (3.5%) found in a previous study in wetlands throughout 12 African countries, including several neighbouring countries of Nigeria [274]. Many factors including the year, season, location, species and age of birds influence prevalence of AIV as is known from wild birds in Europe and America [305-309].

Phylogenetic analyses revealed that all genes of our LPAI H5N2 viruses were most closely related to genes circulating in the Eurasian wild bird influenza gene pool. It has been shown that migratory birds can carry LPAI viruses from one continent to another [77; 310] introducing new genes and mixing gene pools [311-313]. Three main migratory flyways link Africa to Eurasia – the East Atlantic flyway, the Black Sea/Mediterranean flyway and the East Africa/West Asia flyway [77] – and Nigeria is located at the intersection of them. Thus low pathogenic avian influenza genes may have been introduced from Eurasia to Africa by migratory birds. However, Spur-winged Geese are in principle a sedentary sub-Saharan species, normally making only short daily flights, with only rare longer flights, depending on the availability of water. The species is wide-spread throughout sub-Saharan Africa, but does not leave the continent [280; 314]. During the dry season, Spur-winged Geese are highly gregarious around permanent waterbodies [314] such as in the Hadejia-Nguru Wetlands where they mix with Afro-tropical birds and Eurasian migratory birds such as Garganey (*Anas querquedula*), Northern Pintail (*Anas acuta*) or Ferruginous Duck (*Aythya nyroca*) [279; 280; 314]. The mingling of Eurasian migratory and African bird species in the Hadejia-Nguru Wetlands seems to be reflected also in the AIV gene pools.

Interestingly, the NS sequences of the three LPAI H5N2 viruses from Nigeria shared a common ancestor with South African and Zambian influenza isolates from 2004 and 2006, suggesting another origin of the NS gene compared to all other genes. Also, the TMRCA of the Nigerian LPAI H5N2 NS genes suggested that the cluster emerged around October 2004 (January 2002 – February 2007; 95% HPD; node I in Figure 13) whereas the mean TMRCAs for the other genes averaged out during late 2006 or 2007 (Table 6). The TMRCA of the African monophyletic cluster, that included the Nigerian, South African and Zambian strains, suggested that a virus was introduced to Africa between 1997 and 2003. This ancestral virus evolved and probably reassorted with other viruses present in the African wild bird population, as already suggested [254; 282; 315]. The NS gene was acquired by viruses that later spread throughout Africa. Ring recoveries have shown that some intra-African migratory birds from South Africa (e.g. Comb Duck *Sarkidiornis melanotos*) sometimes migrate as far north as West Africa [316] and thus would be able to transmit AIV over long distances within Africa. The presence of similar genes over a four year period (2004-2008; Figure 19) and its probable introduction at least seven years ago suggests that AIV can persist in the African bird population.

The Eurasian-African AIV interphase resembles the situation in the Bering Strait where interregional transmission of influenza viruses occurs between North American and Eurasian birds. Genes from the Asian lineage have been found in Alaska [312; 317] and genes from the American lineages have been found in Japan [318], producing a variety of reassortants. Also, similarly to the persistence of Eurasian genes in Africa, now constituting the African gene pool, H6 genes from the Eurasian lineages have been introduced into North America, gradually replacing the American H6 strains [319].

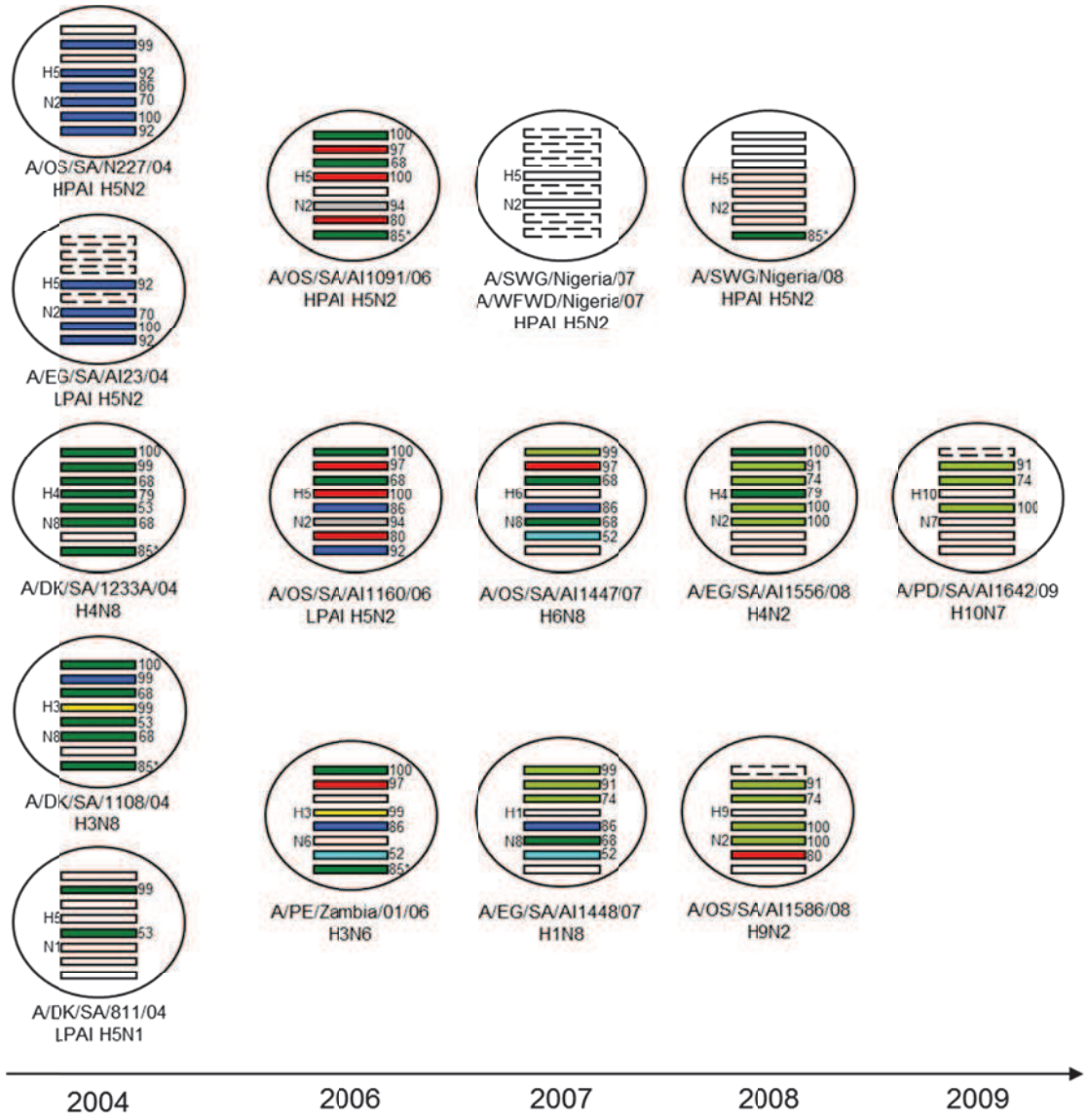

Figure 19. Genetic relationship of African AIV viruses other than HPAI H5N1 viruses sequenced since 2004 by years in wild and domestic birds. Genes have the same colour code if their share a direct common ancestor. Unsequenced genes are indicated as dotted bars and genes that have no African sister gene are shown as white bars. Phylogenetic relationships were first assessed by comparing the African strains with all avian influenza strains downloaded on NCBI Influenza Virus Resource, but based on shorter fragments, depending on the shortest sequences available for the African strains. Representative strains were selected for each gene based on these preliminary analyses and trees were calculated using MEGA4 [290] with Neighbour-Joining method, using the Kimura 2-parameters model and 1000 bootstrap replicates. The star indicates that the values correspond to the probability values from Figure 13. The Figure suggests, based on the available data (from South Africa, Nigeria and Zambia), that some avian influenza viruses may be maintained in the African wild bird population where reassortment events can occur and that these viruses can be transmitted from wild to domestic birds. Only A/DK/SA/811/04 (LPAI H5N1) does not share any gene with a domestic bird strain.

Abbreviations: OS = ostrich, EG = Egyptian goose, SWG = Spur-winged Goose, WFWD = White-faced Whistling Duck, PD = Pekin Duck, PE = pelican, SA = South Africa.

The presence of LPAI H5N2 viruses in African wild birds represents a certain risk also for poultry. Infected wild birds, such as Spur-winged Geese, may introduce LPAI viruses into free-ranging domestic ducks reared in the wetlands in Northeastern Nigeria with whom they occasionally mix [320]. Similar situations have already been observed in South Africa where wild birds were suspected to have infected domestic birds with whom they shared similar genes (Figure 19). Farmed ostriches were infected with H6N8 in 1998 [236] and 2007 [321], with H5N2 viruses in 2004 and 2006 [254; 283], with H9N2 in 2008 and H10N7 was found in domestic ducks [321] (Figure 19). In addition, HPAI viruses are thought to emerge after extensive circulation of H5 (and H7) LPAI subtypes and adaptation in poultry [56; 58; 63; 64]. This is of particular concern since the Nigerian LPAI H5N2 strains belong to a genetic cluster that seems to have an increased propensity to develop the highly pathogenic phenotype. Indeed from a common node (node II, Figure 11) that emerged between June 2002 and May 2004 (95% HPD) highly pathogenic H5N2 strains emerged twice in South Africa in 2004 (not shown in Figure 11 because of its shorter sequence; [236; 254]) and 2006 [236; 254] and in Nigeria in 2007 [272].

Interestingly, the latter Nigerian HPAI H5N2 viruses were found only one year earlier about 10 km away in the same wetlands and in the same wild bird species than the LPAI H5N2 described in this study. For both the HA and NA genes, the HPAI H5N2 formed a sister branch to the LPAI H5N2 but were not the closest known relatives of each other, suggesting that their presence in the wetland resulted from two separate introductions. The question also remains where the Nigerian HPAI H5N2 virus acquired its HPAI phenotype. The wetlands in Northeastern Nigeria provide ample opportunities for cross-species infections and perhaps even the generation of HPAI viruses, normally only after circulation and adaptation in poultry.

In conclusion, we report the presence of LPAI H5N2 viruses in wild birds in an African wetland, that were reassortants with genes from the Eurasian and African gene pools, as a strong evidence of introduction of low pathogenic avian influenza into Africa by Eurasian migratory birds. Furthermore, the circulation of LPAI and HPAI H5N2 strains in wild birds in African wetlands that emerged from a cluster with an increased propensity to develop the highly pathogenic phenotype represents a high risk for poultry especially in areas with low biosecurity that provide opportunities for cross-species infections.

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Chapter 3:

Newcastle disease virus in West Africa - new virulent strains identified in non-commercial farms

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1. Introduction

Newcastle Disease Virus (NDV), or avian paramyxovirus type 1 (APMV-1) (family *Paramyxoviridae*, genus *Avulavirus*, [152]), is the most important virus in poultry [322]. Despite vaccination, APMV-1 causes severe economic losses in poultry farms both in developed and developing countries [239; 323].

APMV-1 strains widely differ in clinical signs and severity of disease that they cause in birds [324]. On the basis of their virulence in chicken, lentogenic, mesogenic and velogenic strains are distinguished. Lentogenic strains cause mild respiratory or enteric infections whereas mesogenic strains induce mainly acute respiratory signs [153]. Some lentogenic and mesogenic strains are used as vaccines [153; 325]. Velogenic strains are responsible for severe disease and high mortality in poultry. Depending on main clinical signs these velogenic viruses are further sub-divided into viscerotropic and neurotropic strains [153]. APMV-1 pathogenicity is largely determined by the sequence of the fusion protein (F protein) cleavage site [175]. The minimum amino acid (aa) motif for virulence in chicken is ^{112}X-R-X-R/K-R-F^{117} [153]. Similar to avian influenza, additional basic amino acids facilitate the proteolytic cleavage of the precursor glycoprotein F0 by ubiquitous proteases and extend cell and organ tropism for enhanced viral replication [326; 327].

The non-segmented single stranded, negative sense RNA genome encodes six proteins. Like many other RNA viruses which have an RNA polymerase that lacks proof-reading, APMV-1 has evolved into genetically highly diverse lineages [72]. Aldous *et al.* [186] proposed six phylogenetic lineages, further subdivided into 13 sublineages. Lineages 1 and 6 contain mainly avirulent viruses, while most virulent viruses are found in lineages 3, 4 and 5. Lineage 2 includes both avirulent and virulent strains. While some lineages seem to be geographically constrained others circulate worldwide [186]. On the African continent outbreaks are rampant but only few strains from Mozambique, South Africa and Uganda have ever been investigated [214; 218; 243].

While small backyard farms are common throughout West Africa, large, highly professional commercial farms are found mainly in Nigeria where the poultry industry is only second to the oil industry [124]. Poultry farming provides secure jobs and is essential for the subsistence of many small producers throughout much of Africa [328]. It is also the main source of high quality protein for human consumption. While the larger farms in West Africa tend to vaccinate against APMV-1 subsistence farmers normally do not. Despite the threat to

poultry farming, APMV-1 has never been characterized on a molecular level throughout West Africa and most of Africa.

In the present study, the genetic diversity of APMV-1 strains from Nigeria, Niger, Burkina Faso and Cameroon was investigated. On the basis of partial F gene sequences we identified strains of different levels of virulence including three new subgroups within lineage 5 with virulent cleavage sites. This is the first molecular epidemiology study for APMV-1 in West Africa and throughout most of Africa.

2. Material and methods

2.1 Sample collection

Between 2002 and 2006, tissues samples (lung, liver or bursa of Fabricius) or cloacal swabs were collected from chickens in commercial farms and slaughterhouses, located in three different South-Western States of Nigeria (Ekiti, Ogun and Oyo States). In 2006 and 2007, cloacal, pharyngeal and tracheal swabs were collected in backyard farms and live bird markets in Nigeria (Sokoto and Oyo States), Niger, Burkina Faso and Cameroon. Most samples were from chickens but some were also obtained from turkeys, ducks, a fowl, a pigeon and a parrot.

2.2 RNA extraction and RT-PCR

All tissues were stored at -20°C until RNA extraction. Fifteen to 50 mg of tissue were homogenized in phosphate-buffered saline using a manual tissue grinder (Bioblock, Tournai, Belgium). To avoid cross-contamination, grinders were disinfected with sodium hypochlorite (10%), and rinsed with double distilled water and 70% isopropanol before use. Cloacal, pharyngeal and tracheal swabs were stored at -80°C in 500 µl of virus transport medium before RNA isolation. RNA was extracted from 140 µl of tissue homogenates or 140 µl of virus transport medium using the QIAamp Viral RNA Mini Kit (Qiagen, Venlo, NL) or from 50 µl of virus transport medium using the MagMAX^{TM}-96 AI/ND Viral RNA Isolation Kit (Ambion, Austin, Texas, USA) with Thermo Electron's KingFisher (Thermo Fisher, Waltham, Massachusetts, USA) following the manufacturers' instructions.

Reverse transcription was carried out for 80 min at 55°C in a 20 µl reaction containing 200 U of SuperScript^{TM} III Reverse Transcriptase, 40 U of RNaseOUT^{TM} Recombinant

Ribonuclease Inhibitor, 5mM DTT, 1x First-Strand Buffer, 0.5 mM dNTP mix, 7.5 mg/L of random nucleotide hexamers (Invitrogen, Merelbeke, Belgium), 2 µl of distilled water and 5 µl of extracted RNA.

APMV-1 positive specimens were detected as a 280 bp fragment of the APMV-1 fusion protein gene (F gene) by nested PCR, using previously published gene-specific primers [329]: FOP1 (5'-TACACCTCATCCCAGACAGGGTC-3') and FOP2 (5'- AGGCAGGG GAAGTGATTTGTGGC-3') at a concentration of 0.1 µM for the first round and FIP1 (5'-TACTTTGCTCACCCCCCTT-3') and FIP2 (5'-CATCTTCCCAACTGCCACT-3') at a concentration of 0.5 µM for the nested PCR. Longer PCR products (550 bp), including the start codon of the F gene were generated for a subset of viruses by semi-nested PCR using other published primers: M610 (0.5 µM; 5'-CTGTACAATCTTGCGCTCAATGTC-3') and F581 (0.5 µM; 5'-CTGCCACTGCTAGTTGTGATAATCC-3') were used in a first round [218] and P1 (0.5 µM; 5'-ATGGGCYCCAGAYCTTCTAC-3', [208]) and F581 (0.5 µM) in the second round.

All PCR reactions were performed on a Mastercycler Gradient (Eppendorf, Hamburg, Germany) in a total volume of 25 µl including 2 to 4 mM $MgCl_2$, 1x PCR buffer, 0.2 mM dNTPs, 0.5 to 1 U Platinum Taq (Invitrogen, Merelbeke, Belgium) and 0.5 µl of cDNA or first round PCR product.

PCR reactions were carried out using the same cycling conditions: initial denaturation at 95°C for 5 min, 40 cycles of amplification at 95°C for 30 s, 58°C for 30s and 72°C for 1 min, and a final extension at 72°C for 10 min. PCR product sizes were visualized by UV illumination in 1.5 % agarose gels stained with ethidium bromide as compared to the 1kb+ size marker (Invitrogen, Merelbeke, Belgium).

2.3 Sequencing and phylogenetic analysis

PCR products were purified using the JetQuick PCR Purification Spin kit (Genomed, Loehne, Germany). Partial F gene sequencing was performed in both orientations using the Big Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems) and (semi-) nested PCR primers. Nucleotide sequences were determined on an ABI 3130 Avant capillary sequencer (Applied Biosystems). Electropherograms were analysed with BioEdit [260] and sequences were aligned using ClustalW [291]. Phylogenetic trees were drawn with Mega v3.1 [330] using the neighbor-joining Kimura 2-parameter model. We consistently used the APMV-1 strain

nomenclature proposed by Aldous *et al.* [186]. In Figure 20 and Figure 21 also the nomenclature proposed by Ballagi-Pordany *et al.* [187] and other authors [190; 214; 219; 233] is shown.

3. Results

A total of 44 partial F gene sequences were phylogenetically compared with representatives of the six known genetic lineages of APMV-1 circulating worldwide [186]. Phylogenetic analysis was based on a 242 nucleotides (nt) sequence of the APMV-1 F gene (nt 332 –573) obtained with primers FIP1 and FIP2 [329]. In the phylogenetic trees, sequences clustered with representative viruses of lineages 1, 2, 3, 4 and 5 (Figure 20). In addition, the genetic grouping was confirmed for 14 representative strains with a longer sequence (nt 67 – 420) located in the region recommended by Aldous *et al.* [186] for phylogenetic analyses (Figure 21). The latter gene segment could only be amplified from a subset of strains, most probably because of suboptimal storage and partial RNA degradation in the other samples. The longer sequence was obtained from at least one representative strain of each (sub-)lineage, including the new ones, described in this study.

Lineage 1. Three APMV-1 sequences originating from chicken samples from Cameroon 2007 and Nigeria 2005 and 2006 clustered with lineage 1. They differed from each other by three or four nt and from the asymptomatic enteric Queensland V4/66 vaccine strain by four or five nt and two or three aa (Figure 20).

Lineage 2. All lineage 2 sequences (12 from Nigeria, 2006/2007; five from Burkina Faso, 2006) had a maximum distance of two nt from the lentogenic LaSota vaccine variant used in Nigeria and Burkina Faso (Figure 20), 13 of them being identical. One nucleotide substitution was non-synonymous. In addition 16 short sequences (92 nt) from commercial farms and slaughterhouses in Nigeria (2002 to 2005) differed by a maximum of one nt from the LaSota strain (data not shown).

Lineage 3. One sequence from a chicken in Nigeria (2006) clustered within sublineage 3a (Figure 21). It differed by only three nt from the mesogenic Mukteswar vaccine (strain BINLA40166).

Lineage 4. Two sequences of this lineage differing by two nt were obtained from a pigeon and a parrot in Nigeria (2007). They were most closely associated with sublineage 4b viruses

(Figure 21), and differed by 12 nt from the closest published sequences, which were obtained from a pigeon in Belgium [331] and a human in the US [252].

Lineage 5. Twenty-one sequences clustered in three different subgroups (Figure 20) clearly distinct from any of the existing sublineage 5 groups, and were therefore considered as new sublineages. Three sequences from a first subgroup, tentatively named 5f, were obtained in 2007 from live bird markets in Sokoto state (Nigeria), which borders with Niger. In the same year four other viruses from this subgroup were identified in backyard chickens in the south of Niger. Sequences from Nigeria differed by three to eight nt from those from Niger, which were 100% identical. A second new sublineage, tentatively named 5g, included ten sequences. This sublineage was found in backyard chicken farms in Burkina Faso (2006), in ducks kept in backyard farms from Niger (2007) as well as in live bird markets in Nigeria (Sokoto state; 2007). Identical sequences were obtained for all viruses identified in Niger or Burkina Faso, respectively. They differed by two nt from each other and by two to three nt from to the most closely related strain from Nigeria. The four Nigerian sequences of sublineage 5g had a maximum of six nt substitutions compared to each other. The third new subgroup, provisionally named 5h, included four sequences from three chickens and a duck kept in backyards in Burkina Faso in 2006, corresponding to two variants differing by four nt. All new sublineages had a common node with previously established lineage 5 subtypes in both phylogenetic trees based on the 242 nt and 354 nt fragments of the F gene (Figure 20 and Figure 21). The intra- and inter-sublineage diversity of established and new sublineage 5 groups has been assessed after selecting representative strains of each sublineage. The minimal genetic distances between the most closely related strains of the known sublineages 5a to 5e ranged from 2.3% (5a and 5c) to 9.2% (5b and 5d; Table 8). In contrast minimal distances between the latter and new sublineages ranged from 9.4% (5a and 5h) to 15.9% (between 5e and 5g). The minimal distances between the new sublineages 5f to 5h ranged from 11.5% (5f and 5h) and 17.3% (5g and 5h).

Cleavage site. Deduced amino acid sequences of the F protein cleavage site of lineage 1 (GKQGR*L) and lineage 2 (GRQGR*L) strains was indicative of a low virulence for these viruses. In contrast the corresponding sequence motif of lineage 3 (RRQRR*F), lineage 4 (RRKKR*F) and lineage 5 (RRQKR*F or RRRKR*F) strains predicted a high virulence.

Table 8: Minimal Kimura-2 distances (%) between all lineages and between all sublineages of lineage 5 (a to e) and the new groups tentatively named 5f, 5g and 5h. Nomenclature according to Aldous *et al.* [186].

	1	**2**	**3**	**4**			
2	10.78						
3	10.67	16.42					
4	14.00	17.71	7.16				
5	17.07	20.01	9.10	8.54			
	5a	**5b**	**5c**	**5d**	**5e**	**5f**	**5g**
5b	6.58						
5c	2.30	5.95					
5d	5.35	9.23	3.50				
5e	5.03	8.56	4.42	6.96			
5f	12.85	12.02	13.23	14.44	14.84		
5g	14.00	13.67	14.00	15.53	15.91	15.20	
5h	9.41	11.50	10.44	12.56	12.20	11.51	17.27

4. Discussion

Although APMV-1 is well known to circulate and cause massive damage in the local poultry industry [332-335], this is the first report of sequences of APMV-1 from West Africa and throughout most of Africa. Partial F gene sequences from 44 APMV-1 strains were compared to representative sequences of the six genetic lineages proposed by Aldous *et al.* [186]. Phylogenetic analysis revealed that all viruses belonged to either one of the five lineages 1, 2, 3, 4 and 5. All five lineages were detected in Nigeria, while only one or two lineages were found in Niger (lineage 5), Burkina Faso (lineage 2 and 5), and Cameroon (lineage 1). APMV-1 strains clustered in the same way when phylogenetic trees were drawn on longer sequence fragments (nt 67 – 420) or the corresponding short sequences (nt 332 –573). This confirmed that the shorter sequence, which was obtained from all strains, was sufficient to assign the new sequences to previously described lineages of APMV-1.

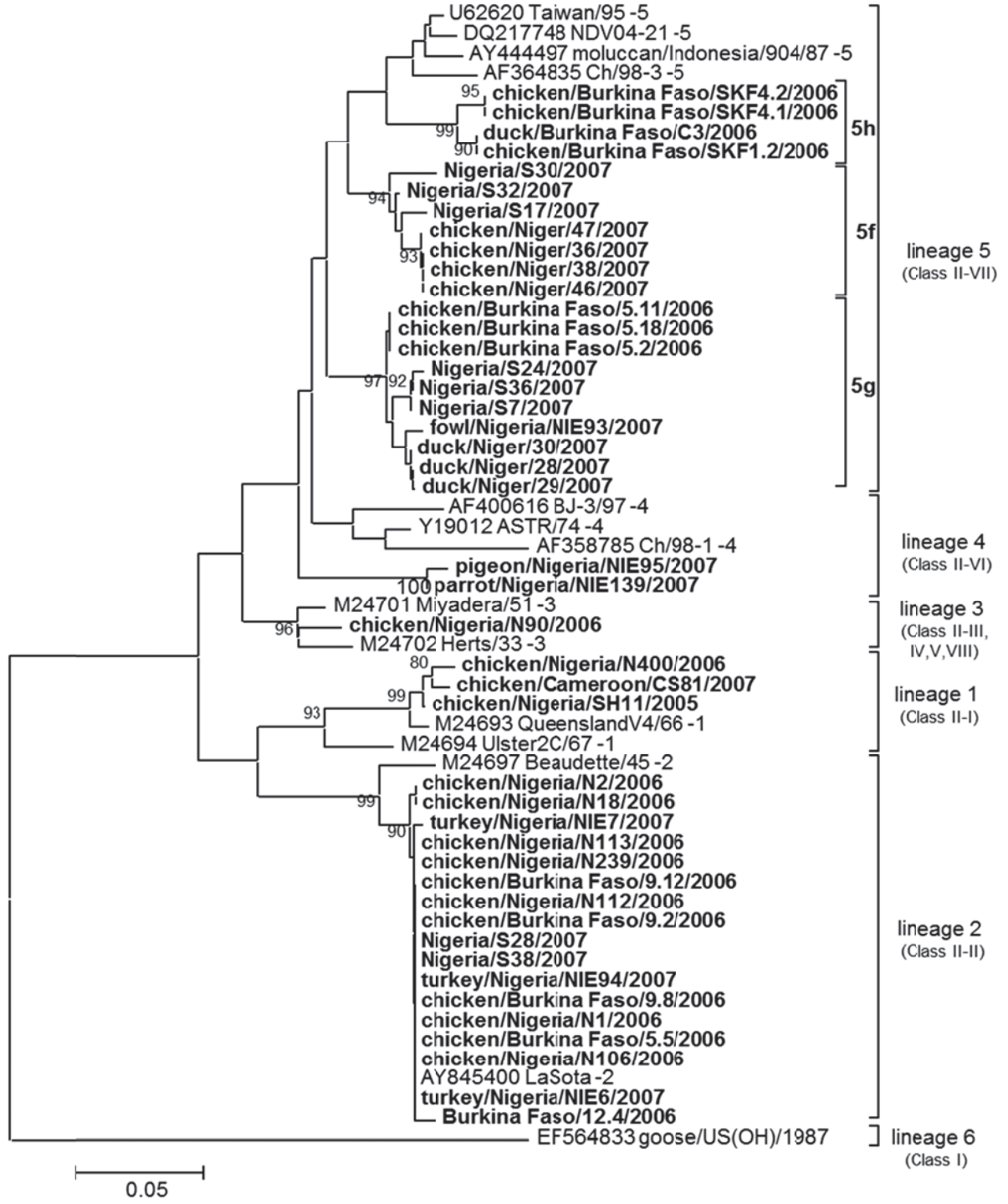

Figure 20. Phylogenetic analysis of 44 partial F gene sequences based on nucleotides 332 to 573 (numbering according to [329]). Fragments were sequenced with FIP1 (nt 313-331) and FIP2 (nt 574-592) primers. Previously published sequences are indicated with their accession numbers. The tree was constructed with the Neighbour-Joining method (Kimura-2 parameter) with 1000 bootstrap replicates. Only bootstrap values higher than 79 are shown. Nomenclatures according to Aldous *et al.* [186] and according to Ballagi-Pordany *et al.* [187] are shown.

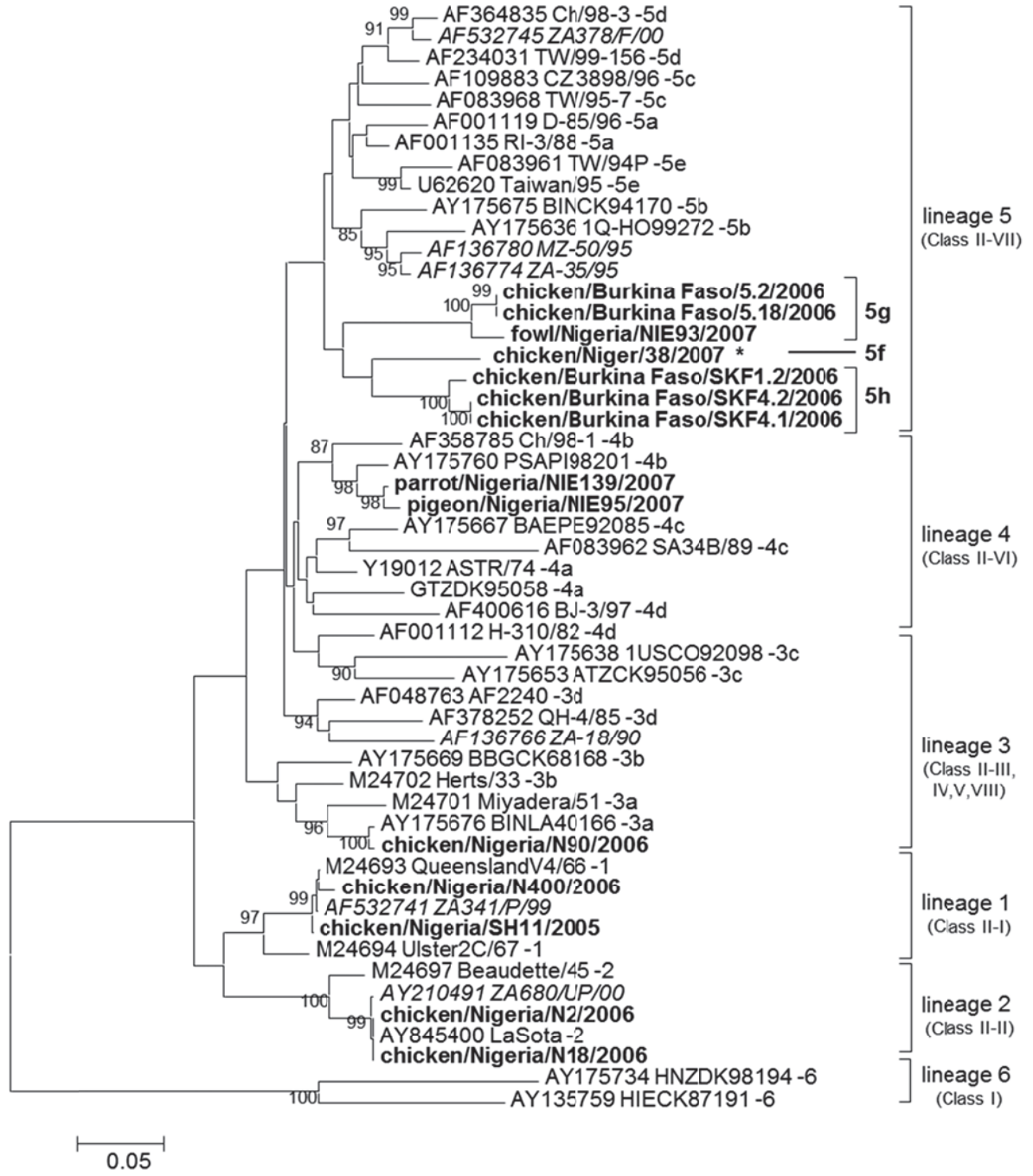

Figure 21. Phylogenetic analysis of 14 partial F gene sequences based on nucleotides 67 to 420 (numbering according to [329]). Fragments were sequenced with P1 (nt 47-66) and F581 (nt 557-581) primers. Previously published sequences are indicated with their accession number. The tree was constructed with the Neighbour-Joining method (Kimura-2 parameter) with 1000 bootstrap replicates. Only bootstrap values higher than 79 are shown. Star indicates that the first 27 nt of the corresponding sequence are missing for this isolate. *Italic* Strains from South Africa (ZA) and Mozambique (MZ) are shown. Nomenclatures according to Aldous et al. [186] and according to Ballagi-Pordany et al. [187] are shown.

All lineage 1 and 2 strains from West Africa had a low virulence predicted on the basis of their F protein cleavage site sequence. To the best of our knowledge no lineage 1 vaccine is currently used in West Africa suggesting that the corresponding viruses are wild-types. In contrast, sequence similarity of lineage 2 viruses with a vaccine strain used in Nigeria and Burkina Faso (Owoade A., Tarnagda Z., pers. comm.) and their apparent high genetic stability in Nigeria during a five year period suggest that these strains are most probably vaccine derived. The single lineage 3 strain described here was genetically similar to the Mukteswar vaccine strain, which is mainly used in South East Asia [336]. In South Africa, lineage 1 and 2 strains were related to vaccines used in the country [218]. However strains of the above lineages were mostly closely related to strains from China, which may be an indication of their origin.

In contrast to lineages 1 to 3, lineage 4 and 5 viruses were clearly wild-type strains with virulent cleavage sites. Sublineage 4b viruses are antigenically distinguishable from all other APMV-1 lineages and are generally referred to as pigeon paramyxoviruses-1 (PPMV-1) [172; 337]. They are mainly found in the Middle East, Asia and Europe [337] including recently in racing pigeons from Finland (www.oie.int/wahid-prod/public.php?page=country_reports). Sublineage 4b viruses from Nigeria were found in a pigeon and a parrot. It is not clear whether these viruses circulate in the country or whether they had been imported by bird trade.

The remaining viruses shared a common node with the different lineage 5 strains. Lineage 5 strains have also been identified in Mozambique and South Africa, and were attributed to sublineages 5b and 5d [214; 218] (Figure 21). However, our lineage 5 strains formed three clusters (supported by high bootstrap values) distinct and genetically distant from all known sublineages and were therefore grouped in three new sublineages 5f, 5g and 5h. The distance matrix shows that most of the minimal genetic distances between the old and the new sublineages as well as minimal genetic distances between the new sublineages of lineage 5 are considerably higher than the minimal distances between the old sublineages. Table 8 even shows that minimal distances of the new sublineages 5 are comparable to distances between lineages 1 to 5 (7.2% to 20%) and possibly warrant the designation of new lineages. However this should be further confirmed by longer sequences.

Moreover, lineage 5 strains from West Africa showed between 10 and 13.75% divergence in the aa sequence deduced from the 242 nt fragment in comparison to the LaSota vaccine strain. One strain (chicken/Niger/38/2007) had an aa substitution (K78R) in a neutralizing

epitope [338]. Although it is generally accepted that APMV-1 exists as a single serotype different levels of cross-protection have been observed in chickens vaccinated with LaSota and challenged with different wild-type strains [249]. The rational of using LaSota as a vaccine in West Africa may thus have to be re-evaluated.

While sublineage 5g viruses were found in Nigeria, Niger and Burkina Faso, sublineage 5h was only detected in Burkina Faso and 5f strains were present in Niger and Nigeria; sequences obtained from different countries were genetically distinct even within sublineages. Interestingly none of the lineage 5 strains from West Africa were found in commercial farms.

The high genetic diversity of lineage 5 strains in West Africa, their common ancestry suggested by the phylogenetic trees, their presence in three different Sub-Saharan countries as well as the absence of similar APMV-1 strains on other continents suggest that lineage 5 strains have been enzootically circulating for many years in West Africa and have apparently not been exported to other continents. While lineage 5 seems to be the indigenous APMV-1 variant to West Africa in the local breeds of backyard farms and possibly in wild birds, the other lineages seem to have different geographic origins including widely used vaccines. The absence of lineage 5 strains in commercial farms suggests that current vaccination programs efficiently protect commercial poultry. In conclusion, this study shows that as geographic surveys become more complete the genetic diversity of APMV-1 seems to further unfold.

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Chapter 4:

Genetic diversity of Newcastle Disease virus in poultry in West and Central Africa

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Manuscript in preparation

1. Introduction

Newcastle disease, caused by virulent Newcastle disease virus (NDV), is one of the most important diseases of poultry worldwide and is recognized as the main pathogen affecting the commercial and traditional poultry industry in West Africa [339]. NDV, a member of the Avulavirus genus in the Paramyxoviridae family [152], is classified into several pathotypes (asymptomatic enteric, lentogenic, mesogenic, viscerotropic or neurotropic velogenic) based on the clinical symptoms observed in chickens [166]. Avirulent and virulent NDV strains are also distinguished based on the sequence of the cleavage site of the fusion (F) protein. During the replication cycle, the fusion protein is translated as a precursor F_0 that must be cleaved by host cell proteases into two F_1 and F_2 subunits for viral particles to be infectious [167]. Most virulent strains exhibit the consensus sequence ^{112}R/K-R-Q-R/K-R*F^{117} in contrast to ^{112}G/E-K/R-Q-G/E-R*L^{117} in avirulent viruses [166; 168]. Additional basic amino acids in the virulent viruses allows the F_0 precursor to be cleaved by proteases, most likely furin-like enzymes, present in a wider range of cells, providing the viruses the ability to replicate in a range of tissues and organs resulting in fatal systemic infection [169]. NDV strains are also genetically diverse and they were classified into lineages based on partial F gene sequences [186] or into two classes and several genotypes based on restriction site mapping, genome length and F gene sequences [156; 187].

The first records of virulent NDV in West and Central Africa date from the 1950s (Hills *et al.*, cited by [238]). Since then, several viruses from different outbreaks have been characterized by pathogenicity tests [335; 340] but the first insight about the molecular feature of these viruses were revealed only recently [241; 289]. Based on partial F gene sequences, we have previously classified West African strains (Niger, Nigeria, Burkina Faso) into three new sublineages 5f, 5g and 5h. Cattoli *et al.* [241] obtained full length F gene sequences from reported NDV cases in six African countries and defined a new lineage 7 divided into 4 sublineages (7a to 7d). Here, we report 96 additional full length F gene sequences of NDV detected during active and passive surveillance in Nigeria, Cameroon, Central African Republic and Côte d'Ivoire between 2006 and 2011, providing further insights into the genetic diversity of the circulating strains. The burst of sequences provided by this study allowed us to confirm the high genetic diversity leading to the definition of three new lineages divided into six sublineages.

2. Materials and Methods

2.1 Sample information

A total of 3610 domestic birds (mainly chickens but also ducks, geese, guinea fowls and turkeys) were sampled in a variety of locations, including free ranging animals, live-poultry markets, backyard and commercial farms between 2006 and 2011 in four countries in West and Central Africa (Table 9, Figure 22). Except in Côte d'Ivoire where samples were collected during passive surveillance, all other samples were collected during active surveillance. The vast majority of collected material consisted of pooled tracheal and cloacal swabs, but cloacal or tracheal swabs, faeces or organs (lung, liver, intestine, trachea, spleen and brain) were also collected.

Figure 22. Collection sites in Côte d'Ivoire, Nigeria, Cameroon and Central African Republic. The localization of provinces or states visited are indicated by black circles. In Côte d'Ivoire, the following provinces were: 1. Savanes; 2. Worodougou; 3. Sud Bandama; 4. Zanzan; 5. Moyen Comoé; 6. Agnéby; 7. Bas Sassandra; 8. Sud Bandama; 9. Lagunes.

2.2 RNA extraction and detection PCR

All swabs and fecal samples were discharged in 500 µl of virus transport medium (VTM; [258]). RNA was purified from 140 µl of VTM with QIAamp Viral RNA Mini Kit (Qiagen, Venlo, The Netherlands) or from 50 µl of virus transport medium using the MagMAX^{TM}-96 AI/ND Viral RNA Isolation Kit (Life Technologies, Merelbeke, Belgium) with Thermo Electron's KingFisher (Thermo Fisher, Waltham, Massachusetts, USA) following the manufacturers' instructions. Approximately 30 mg of organs were homogenized with

stainless steel beads (Qiagen) and TissueLyser II (Qiagen) in 600 µl of lysis buffer of RNeasy Mini Kit (Qiagen) and RNA was extracted according to the manufacturer's protocol. Reverse transcription, first round and nested PCRs for NDV detection were performed as described previously [289] on the 3610 samples included in the study.

Table 9. Distribution of collected and positive samples according to countries, years of sampling and species.

Country	Year	Number of positive sample/Number of sample tested by species					
		chicken	duck	goose	guinea fowl	turkey	not specified
Cameroon	2009	4/644					
	2011	12/452					
Central African Republic	2008	3/88					
Côte d'Ivoire	2006	1/26	1/7				0/1
	2007	6/13	0/1		0/1	0/1	
	2008	0/1	0/2				
Nigeria	2006	0/49	0/15		0/6	0/1	0/6
	2007	1/185	0/17		0/1	0/6	1/24
	2008	5/558	0/177	0/9	0/15	1/28	0/1
	2009	69/576	0/72		3/51	2/42	
	2011	46/484	0/5		1/44	1/1	
TOTAL		147/ 3076	1/296	0/9	4/118	4/79	1/32

2.3 Sequencing PCR for partial and full F gene

Partial and full F gene sequences were obtained by several overlapping fragments generated by one-step followed by (semi-)nested PCRs. All primer used and designed in this study are listed in Table 10. One-step PCRs were performed with OneStep RT-PCR Kit (Qiagen) in a final volume of 25 µl including 1 µl of enzyme mix, 1.25 mM MgCl2, 0.4 mM dNTP, 4 U RNase OUT (Life Technologies), 0.5 µM of each primer and 2 µl of RNA. Nested PCRs were carried out with 1 U Platinum® Taq DNA Polymerase (Life Technologies), 4 mM MgCl2, 0.2 mM dNTP, 0.1 µM of each primer and the equivalent of 0.5 µl of first round PCR product. Reverse transcription and amplification were done at 50°C for 30 min, 95°C for 15 min, 40 cycles at 95°C for 30 s, 54 to 56°C for 30s, 72°C for 60 to 90 s, and a final elongation at 72°C for 10 min. Similar cycling conditions were used in the nested reactions.

Table 10. Sequences of primers used to amplify and sequence partial or full F genes.

Name	Orientation	Sequence	Localization	Reference
M-3701f	Forward	CTGCARAGCTGTADGGTTGTG	M gene	this study
M610	Forward	CTGTACAATCTTGCGCTCAATGTC	M gene	[218]
P1	Forward	ATGGGCYCCAGAYCTTCTAC	F gene	[208]
M-4650-R	Reverse	AAGAGGCCTGCCRTCAA	F gene	[241]
F581	Reverse	CTGCCACTGCTAGTTGTGATAATCC	F gene	[218]
F-4639f	Forward	TGAYGGCAGGCCTCTT	F gene	this study
F-4932f	Forward	CAACCGCTGCACAGATAA	F gene	this study
F-4954f	Forward	AGCTGCGGCYCTRATACAA	F gene	this study
F-5042f	Forward	GAGGTCACYGACGGATTAT	F gene	this study
F-1258-R	Reverse	ACATTGCATGAWTGTCTRTC	F gene	[241]
F-5488f	Forward	TCAGCACTTGTCCCAAA	F gene	this study
F-5447-R	Reverse	AGGTGGCACGCATRTTATT	F gene	[241]
F-5566r	Reverse	CAGTATGAGGTGTCAAGTT	F gene	this study
F-5716f	Forward	GTCATTGCCAATTGYAAGATAA	F gene	this study
F-5749f	Forward	AGACCCTCCAGGYATCA	F gene	this study
F-5888f	Forward	GGCTCAGTGGGGAAT	F gene	this study
F-5968r	Reverse	AYTGCTTATTGAATTGTTGACATT	F gene	this study
F-6086f	Forward	GGTACACTTAGCCTGRTHTT	F gene	this study
F-6146r	Reverse	CTTYTGTTGCGCCTTT	F gene	this study
F-6398r	Reverse	GATGCGGTAGAACGGATGTT	HN gene	this study
F-7979-R	Reverse	AGRGCCACYTGCTTRTATA	HN gene	[241]

2.4 Sequencing and sequence assembly

All PCR products were visualized on a 1.5% agarose gel stained with SYBR® Safe DNA gel stain (Life Technologies) and purified using JetQuick PCR Purification Spin kit (Genomed, Loehne, Germany) or QIAquick Gel Extraction Kit (Qiagen) when multiple bands were visible. Sequencing was performed in both orientations using the Big Dye Terminator v.3.1 cycle sequencing kit (Life Technologies) and ABI 3130 Avant capillary sequencer (Applied Biosystems). Primers used for generating the PCR fragments were used in the sequencing reaction, and for longer PCR products, additional internal primers were used (Table 10).
Contigs were assembled using SeqScape v2.5 (Applied Biosystems) and analyzed with BioEdit [260]. The following nomenclature was used: host/country/strain number/year, but strains obtained in this study are referred to by their strain number in the text.

2.5 Lineage classification

All complete fusion gene sequences available on GenBank (as of August 2012) were aligned using ClustalW [291]. Sequences with insertions or deletions resulting in frame shifts were

cured from the dataset. Recombinant sequences were identified with the following methods (RDP, GENECONV [341], MAXCHI, CHIMAERA [342], BOOTSCAN [343], Sister Scanning [344], and 3SEQ [345] as implemented in RDP3 software [346]. Sequences were identified as true recombinant when p value was < 0.001 for at least two tests. All recombinant sequences were removed from the dataset, as well as the recombinants identified based on complete genome analyses by Chong *et al.* [347] and Diel *et al.* [348]. The final dataset contained 812 previously published sequences and 96 obtained in this study.

Lineage and sublineages were determined based on the tree topology, the bootstrap values and the mean evolutionary distances between lineages. Distances were calculated by pair-wise analysis with Neighbour-Joining Kimura-2 parameter model and 1000 bootstrap replicates as implemented in MEGA v5.03 [261]. The mean interpopulational diversity was used to determine the cut-off value between lineages. For these analyses, lineage 6 (112 strains) was excluded due to its much higher genetic distance (between 0.35 and 0.39 mean genetic distance) to lineages 1 to 5. The selected criteria to define a lineage were: grouping supported by a bootstrap value > 70% and between group distance ≥ 0.1.

2.6 Phylogenetic analyses

Kimura distances were calculated according to the Kimura 2-parameter model on partial (375 nt) or complete (1662 nt) F gene sequences. Phylogenetic relationships were inferred by comparing the sequences obtained in this study with all complete F gene sequences available on GenBank, lineage 6 strains included. Trees were calculated with the Neighbour-Joining method, using the Kimura 2-parameter model and 1000 bootstrap replicates (MEGA v5.03). 908 sequences were included to calculate the tree presented in Figure 23, only a few representative strains with a 375 nt length were used to calculate the tree presented in Figure 24. Figure 25 is a detailed view in of lineages 7, 8 and 9 aggregated in Figure 23.

3. Results

Between 2006 and 2011, a total of 3610 samples were collected and screened for the presence of NDV (lineage 6 excluded). Chickens were exclusively sampled in Cameroon and in Central African Republic (Table 9). Chickens, ducks guinea fowl and turkeys were sampled in Côte d'Ivoire and Nigeria, and geese were also sampled in Nigeria. The species origin of 32 samples was not determined (Table 9). In Nigeria, chickens were more often infected by

NDV (6.5%, *p*-value < 0.001), compared to turkeys (5.1%) and guinea fowls (3.4%; Table 11), whereas ducks had a significant lower chance of being infected (*p*-value < 0.001). Birds sampled in the Nigerian live-poultry markets were statistically more often infected with NDV (10%; *p*-value < 0.001), compared to the other locations. On the other hand, free-ranging birds had a lower chance of being infected (*p*-value < 0.001).

Table 11. NDV prevalence in bird species from various sampling locations in Nigeria

sampling location	chicken		duck		goose		guinea fowl		turkey		total	
	No. pos/No. tested	%	No. pos/No. tested	%	No. pos/No. tested	%	No. pos/No. tested	%	No. pos/No. tested	%	No. pos/No. tested	%
market	99/873	11.3	0/74	0			4/90	4.4	3/19	15.8	106/1056	10
backyard farm	11/204	5.4	0/17	0			0/12	0	0/28	0	11/261	4.2
commercial farm	6/115	5.2							0/3	0	6/118	5.1
free ranging	5/638	0.8	0/151	0	0/7	0			1/9	11.1	6/805	0.7
unknown	0/22	0	0/44	0	0/2	0	0/15	0	0/19	0	0/102	0
Total	121/1852	6.5	0/286	0	0/9	0	4/117	3.4	4/78	5.1	129/2342	5.5

3.1 Genetic classification

Sequencing of the fusion gene was attempted for all positive samples (*n*=157), but 96 full and 5 partial sequences were obtained. These sequences were used for assessing genetic classification and for epidemiologic investigations.

Each strain of the dataset consisting of 908 complete F gene sequences was attributed to a lineage based on the tree topology, bootstrap values and previous classifications. Groups 3a, 3b, 3c, 3d, 3e and 3g previously considered as one lineage by Aldous *et al.* [186] based on the classification fn partial (375 nt) sequences were here regarded as separate lineages due to their genetic diversity and their polyphyly (Figure 23). Also group 5b, previously considered as a sublineage of lineage 5, did not constitute a monophyletic group together with the other sublineages 5d, 5c, 5e (Figure 23). However, the same denomination was kept to avoid confusion.

The cut-off value to assign a group of sequences to a lineage was set to 0.1 (±0.005) mean interpopulation diversity, which corresponds to the mean evolutionary distance between lineages. Mean evolutionary distances between previously defined lineages, i.e. 1 to 5 were

all higher than 0.1, except between lineages 1 and 3b, 1 and 3e, 3a and 3b, 3a and 3e, 3b and 3d or 3b and 3e (Table 12).

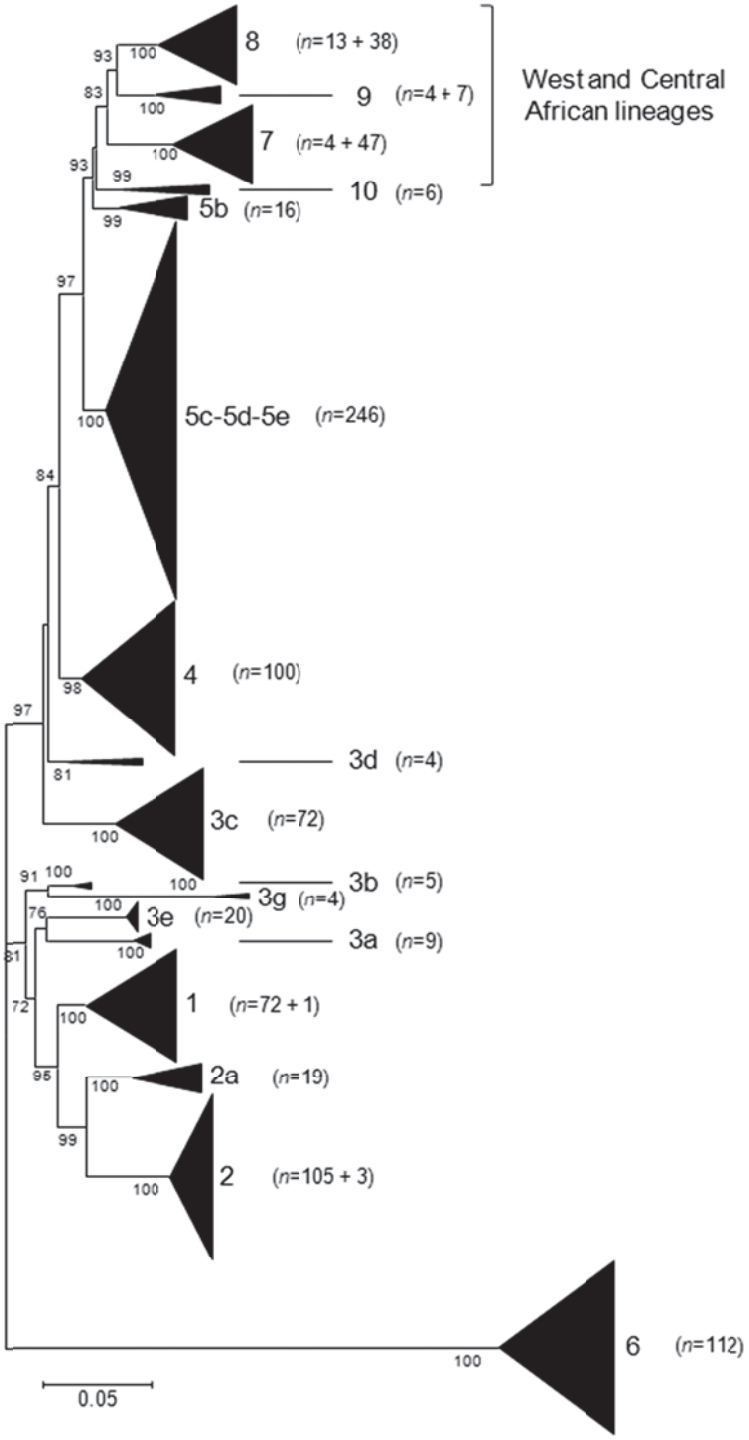

Figure 23. Overview of NDV lineages (complete sequences) defined in this study. Only bootstrap values ≥ 50% are shown. The scale corresponds to number of base substitutions per site. The number of sequences included in each lineage is indicated in brackets as the number of previously published sequences + the number of sequences obtained in this study.

Table 12. Estimates of evolutionary divergence over sequence pairs between lineages (lineage 6 excluded)

	1	2	2a	3a	3b	3c	3d	3e	3g	4	5	7	8	9	10
1		[0,008]	[0,007]	[0,007]	[0,007]	[0,010]	[0,009]	[0,007]	[0,010]	[0,010]	[0,010]	[0,011]	[0,010]	[0,010]	[0,011]
2	0,116		[0,008]	[0,009]	[0,009]	[0,011]	[0,010]	[0,009]	[0,011]	[0,010]	[0,011]	[0,012]	[0,012]	[0,011]	[0,012]
2a	0,106	0,109		[0,010]	[0,009]	[0,011]	[0,010]	[0,009]	[0,011]	[0,011]	[0,011]	[0,012]	[0,012]	[0,012]	[0,012]
3a	0,104	0,129	0,126		[0,007]	[0,010]	[0,009]	[0,007]	[0,010]	[0,010]	[0,010]	[0,011]	[0,011]	[0,011]	[0,011]
3b	0,093	0,118	0,114	0,077		[0,009]	[0,007]	[0,007]	[0,008]	[0,008]	[0,009]	[0,010]	[0,010]	[0,010]	[0,011]
3c	0,172	0,185	0,186	0,165	0,138		[0,008]	[0,011]	[0,012]	[0,008]	[0,008]	[0,009]	[0,009]	[0,009]	[0,009]
3d	0,132	0,150	0,147	0,125	0,094	0,126		[0,009]	[0,010]	[0,007]	[0,008]	[0,008]	[0,009]	[0,009]	[0,008]
3e	0,099	0,118	0,115	0,087	0,075	0,158	0,118		[0,010]	[0,010]	[0,010]	[0,011]	[0,011]	[0,011]	[0,011]
3g	0,172	0,192	0,187	0,163	0,116	0,199	0,172	0,152		[0,011]	[0,011]	[0,013]	[0,011]	[0,011]	[0,013]
4	0,160	0,177	0,174	0,159	0,124	0,147	0,112	0,151	0,197		[0,008]	[0,008]	[0,009]	[0,008]	[0,008]
5	0,158	0,183	0,173	0,154	0,126	0,146	0,116	0,148	0,203	0,120		[0,008]	[0,008]	[0,008]	[0,007]
7	0,191	0,216	0,199	0,194	0,162	0,173	0,143	0,189	0,238	0,151	0,131		[0,008]	[0,008]	[0,008]
8	0,161	0,191	0,182	0,166	0,139	0,157	0,132	0,155	0,196	0,136	0,119	0,122		[0,007]	[0,008]
9	0,166	0,187	0,180	0,164	0,135	0,154	0,127	0,154	0,198	0,126	0,113	0,124	0,100		[0,007]
10	0,166	0,190	0,176	0,160	0,136	0,157	0,118	0,157	0,211	0,120	0,108	0,124	0,114	0,106	

The number of base substitutions per site from averaging over all sequence pairs between lineages are shown. Results were based on the pair-wise analyses of 796 nucleotide sequences and the number of sequences analysed by lineages were: 1, *n*= 73; 2, *n*=108, 2a, *n*=19; 3a, *n*=9; 3b, *n*=5; 3c, *n*=72; 3d, *n*=4, 3e, *n*=20; 3g, *n*=4; 4, *n*=100; 5, *n*=262; 7, *n*=51; 8, *n*=52; 9, *n*=11; 10, *n*=6. Analyses were conducted using the Kimura 2-parameter model with 1000 bootstrap replicates in MEGAv5 (standard error estimates shown between squared brackets). Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1662 positions in the final dataset.

Based on the same criteria, sequences from West and Central Africa were attributed to 3 new lineages tentatively named lineages 7, 8 and 9 (Table 12). Each one of these new lineages was also divided into two sublineages due to their high genetic diversity (Table 13). Evolutionary distances between the newly defined sublineages were comparable to distances between sublineages 4a, 4b and 4d and 5b, 5c and 5d. Genetic distance between sublineages 5b and 5d was higher than the cut-off value for subdivision into lineages, suggesting that group 5b may need to be promoted to a lineage. In addition, a lineage 10 was also defined and included a sequence from an outbreak in in Peru in 2008 [349] and goose isolates from China from 2010 and 2011.

The same classification was performed on the 375 first nucleotides of the same dataset, including the only three partial sequences of previously named sublineage 5h (data not shown). This analysis places sublineage 5h as a sister clade to 7a and 7b, suggesting a possible sublineage 7c. Further classification based on complete F gene sequence of "5h" is warranted in order to confirm its grouping.

Table 13. Estimates of evolutionary divergence over sequence pairs between sublineages

Sublineages of lineage 4

	4a	**4b**	**4d**
4a		[0.006]	[0.006]
4b	0.097		[0.005]
4d	0.093	0.087	

Sublineages of lineage 5

	5b	**5d**	**5c**
5b		[0.006]	[0.006]
5d	0.101		[0.004]
5c	0.087	0.045	

Sublineages of lineage 7

	7a	**7b**
7a		[0.006]
7b	0.072	

Sublineages of lineage 8

	8a	**8b**
8a		[0.006]
8b	0.075	

Sublineages of lineage 9

	9a	**9b**
9a		[0.006]
9b	0.061	

The number of base substitutions per site from averaging over all sequence pairs between lineages are shown. The number of sequences analysed by sublineages were: 4a, *n*=7; 4b, *n*=80; 4d, *n*=12; 5b, *n*=16; 5c, *n*=7; 5d, *n*=237; 7a, *n*=17; 7b, *n*=34; 8a, *n*=49, 8b, *n*=3; 9a, *n*=4; 9b, *n*=7. Analyses were conducted using the Kimura 2-parameter model with 1000 bootstrap replicates in MEGAv5 (standard error estimates shown between squared brackets). Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 1662 positions in the final dataset.

3.2 Phylogenetic analyses

Vaccine-like strains (based on 375nt). In Cameroon, two strains from Cameroon from 2011 clustered in lineage 1, and were similar to Queensland V4 (Kimura distance 0.3%, 375nt). Strain CAE11-855 had also a genetic distance of 0.8% (242 nt) to another isolate from Cameroon (chicken/Cameroon/CS81/2008; FM200839; data not shown). Three strains from 2009 and four from 2011 clustered in lineage 2 and were very similar to the vaccine strain B1 (JN872150; Kimura distance 0 to 0.5%, 375 nt), suggesting that all lineage 1 and 2 strains were linked to the use of live vaccines (Figure 24).

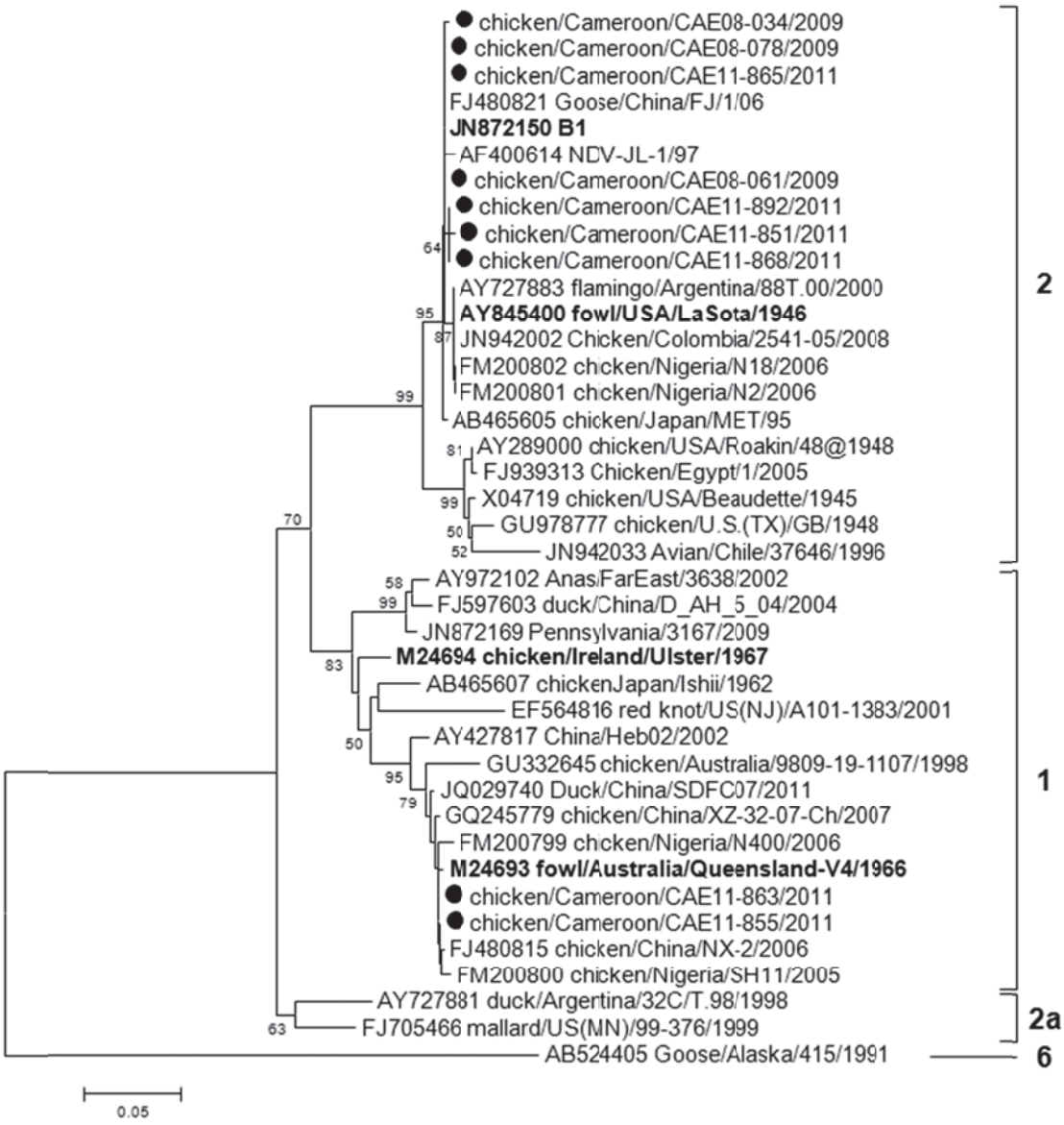

Figure 24. Phylogenetic analyses of lineage 1 and 2 strains based on partial (375nt) F gene sequences. Sequences obtained in this study are indicated with a black circle. Vaccine strains are indicated in bold. Only bootstrap values ≥ 50% are shown. The scale corresponds to number of base substitutions per site.

Lineage 7. Fourteen strains from three Nigerian States (Sokoto, Yobe, Lagos) sampled in 2007, 2009 and 2011 clustered in sublineage 7a, previously named 5f [289], together with two strains from Niger from 2006 and one from Nigeria from 2008 (Figure 25A). Thirty-three strains from Nigeria (Sokoto, Yobe, Benue; 2008, 2009, 2011) sequenced in this study clustered in sublineage 7b together with another strain from Katsina State from 2007 (chicken/Nigeria/VRD07-233/2007). Interestingly, the three strains NIE09-1596, NIE09-1599, NIE09-1597 were found in a commercial farm in Benue State and represent one of the very few cases of virulent NDV in commercial farms (Figure 25A).

Lineage 8. Thirty-five strains obtained in this study clustered within sublineage 8a (previously sublineage 5g [289]), the majority of them were sampled in Nigeria (Sokoto, Yobe, Benue; 2007, 2008, 2009, 2011) but also in Cameroon in 2009 and Côte d'Ivoire in 2007. Sublineage 8a is the most geographically dispersed as it was also found in Niger, Cameroon and Burkina Faso in 2008 ([241]; Figure 25B). In the future, this sublineage may need to be further subdivided due to the rather high genetic distance between CAE08-318, the group formed by NIE10-182, NIE07-216, NIE08-A-2199 and NIE08-2261, and the rest of sublineage 8a. Sublineage 8b was formed by three very similar strains detected in a commercial farm in Central African Republic in 2008 (Figure 25B), and constitute the third isolation of virulent NDV in commercial farm in West and Central Africa.

Lineage 9. Two strains from Côte d'Ivoire from 2007 clustered in sublineage 9a, together with one strain from Mauritania from 2006 (Figure 25A). Sublineage 9b comprised three strains from Côte d'Ivoire from 2006 and 2007, two strains from Nigeria from 2011 as well as one strain from Côte d'Ivoire from 2008.

Phylogenetic analyses revealed that several (sub)lineages are found in every country, except in Central African Republic where only sublineage 8b was identified. In Cameroon, nine strains are related to vaccine strains, either B1 or Queensland V4, but one sublineage 8a was also found. Sublineages 8a, 9a and 9b were circulating in Côte d'Ivoire, while sublineages 7a, 7b, 8a and 9a were found in Nigeria (Figure 25 and Figure 26). Also in Nigeria, which was more extensively sampled, several sublineages and several clusters within each sublineage can be found in the same States (Figure 25). For instance, two clusters of sublineage 7a, three clusters of sublineage 7b and five clusters if sublineage 8a were found in Sokoto State. Also sublineages 7a, 7b and 8a were found in live bird markets in Yobe State (Figure 25).

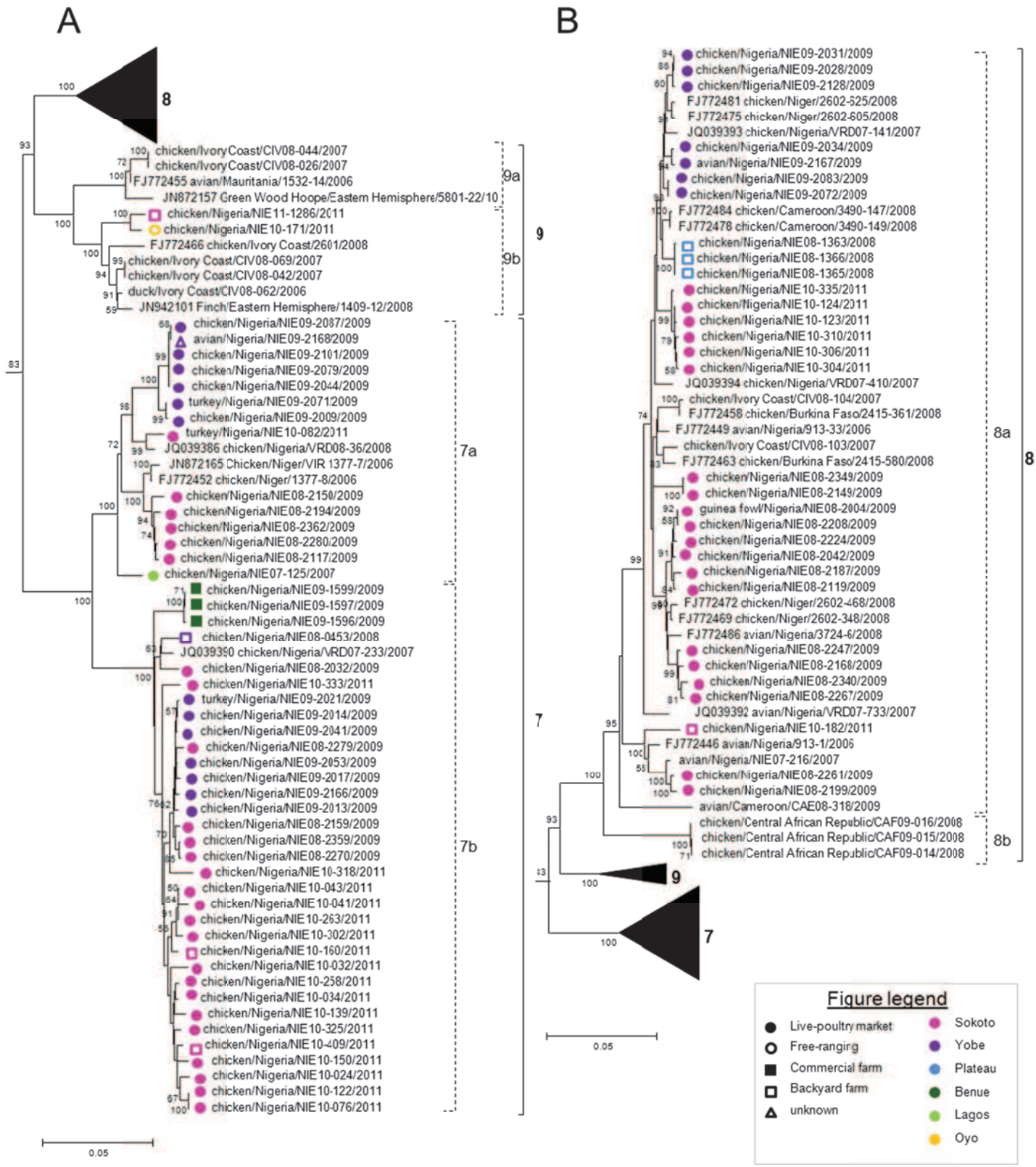

Figure 25. Expanded view of lineages 7 and 9 (panel A) and lineage 8 (panel B) aggregated in Figure 23. Sampling location (State, type of farm/market/free-ranging) are detailed for the samples collected in Nigeria during this study. Accession numbers of previously published sequences available on GenBank are indicated. Only bootstrap values ≥ 50% are shown. The scale corresponds to number of base substitutions per site.

3.3 Analyses of deduced amino acid sequence of the F protein

Deduced cleavage site sequences. Lineage 1 and 2 strains encoded for fusion cleavage site motifs ^{112}GKQGR*L^{117} and ^{112}GRQGR*L^{117} respectively, both typical of avirulent viruses. Deduced amino acid cleavage site sequences of all lineage 7, 8 and 9 strains were indicative of virulent viruses. All strains of sublineages 7a, 8b, 9a and 9b encoded for cleavage site sequence ^{112}RRQKR*F^{117} but CIV08-44 and CIV08-26 (sublineage 9b) had I118V. Two cleavage site motifs were identified in sublineages 7b and 8a. The majority of sublineage 7b strains had a ^{112}RRRKR*F^{117} cleavage site sequence but two groups of strains, namely NIE09-1596/NIE09-1599 /NIE09-1597 and NIE10-034/NIE10-258/NIE10-139/NIE10-325, shared a ^{112}RRQKR*F^{117} sequence. All sequences of sublineage 7b had a I118V substitution. Conversely, all sublineage 8a strains have a ^{112}RRQKR*F^{117} sequence except the group formed by strains NIE10-123/ NIE10-124/NIE10-304/ NIE10-306/ NIE10-310/ NIE10-335 that had a ^{112}RRRKR*F^{117} cleavage site.

Virulent strains were characterized in apparently healthy animals (45/92), in 13/92 sick animals and no information was available for 34/92 birds.

Neutralising epitopes. The fusion protein contains several neutralising epitopes important for its structure and its function [350; 351]. The majority of the strains conserved the residues D72, E74, A75, K78, A79, L343 and the stretch ^{151}ILRLKESIAATNEAVHEVTDG^{171}. However, all lineage 7 strains shared a K78R substitution and five strains had a A79G substitution. The three sublineage 8b strains share A75T and A79T substitutions. Variability was also observed at position 170, where all lineage 9 strains had a D170S substitution but D170G was also found once and D170E found twice. L343P and L343Q were found in one and two strains respectively.

N-glycosylation sites. The F protein contains six conserved predicted N-glycosylation sites (N-X-S/T, where X is any amino acid but a proline) which may play a role in fusion promotion and virus replication. Predicted N-glycosylation site were conserved among all strains (^{85}NRT^{88}, ^{191}NNT^{193}, ^{366}NTS^{368}, ^{447}NIS^{449} or ^{447}NVS^{449}, ^{471}NNS^{473}, ^{541}NNT^{543}) similarly to the other strains published so far, showing that no difference in viral fusion or virulence due to the loss of N-glycans could be expected [352].

4. Discussion

Despite that outbreaks due to NDV occurred in Africa for decades, there was no information about the genetic characteristics of the virulent strains circulating in West and Central Africa until recently. Previous studies by us and others have shown that different strains are co-circulating [221; 238; 241; 289; 353; 354] but the present study provides a more detailed understanding of their genetic diversity, especially in Nigeria, and summarizes their geographic spread as currently known. Based on the comparison of the genetic diversity of complete F gene sequences, we grouped the previously published strains into 12 lineages and we propose that the virulent NDV strains found in West and Central Africa should be classified into three new lineages, each being further divided into at least two sublineages. A similar analysis was performed by Diel *et al.* [348] who determined 15 genotypes (I to XV), the numbering being based on the other classification system concomitantly used [156; 187], and West and Central African strains were classified into a single genotype XIV. The exclusion of recombinant sequences and the fact that lineage 5 was not split up in our analyses explain the discrepancies between the number of lineages or genotypes. Diel *et al.* [348] grouped the West African strains in genotype XIV but only six sequences of this group were included in the analyses at the time. We have shown that, as surveillance progresses, the genetic diversity unfolds and the classification will need constant updates. Additional surveillance in other countries in West and Central Africa will likely reveal higher diversity and the current lineages 7, 8 and 9 may need further subdivision.

In this study, vaccines-related strains were only detected in Cameroon, but none were found in Nigeria, contrarily to a previous study where mainly commercial farms were investigated [289], confirming that the detection of vaccine-related strains is mainly linked to the use of live vaccines, especially in commercial farms. However, sublineage 7b strains were found in a commercial farm in Nigeria where birds were vaccinated against NDV (vaccine strain not specified). Sublineage 8b was also found in a commercial farm in Central African Republic where farms are smaller and where it is not unusual for farmers to sell and buy chickens and mix them with their flocks. It highlights the risk that virulent strains may be introduced into commercial flocks, a high prejudice for the poultry sector.

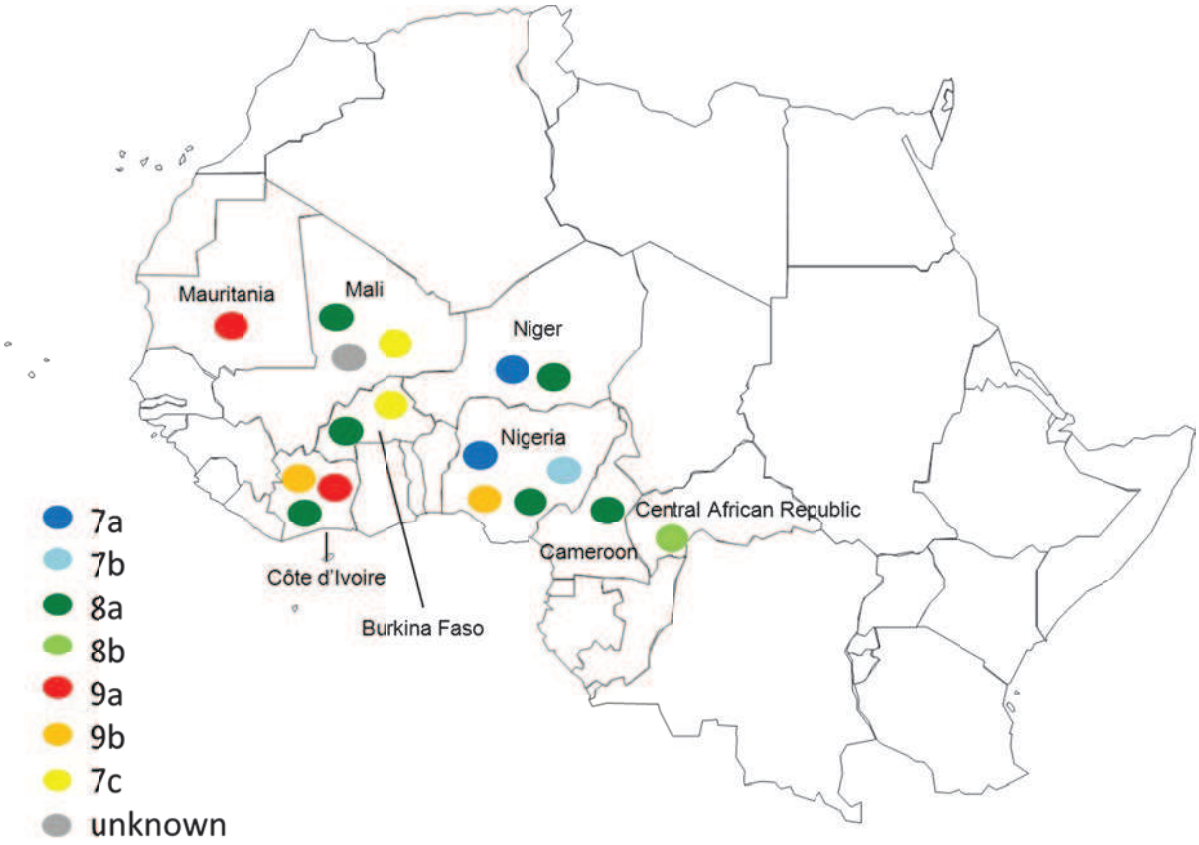

Figure 26. Summary of detection of lineages 7, 8 and 9 in West and Central Africa (including previously published results [241; 289; 353]).

However, in Nigeria, birds sampled in live bird markets were significantly more often positive for NDV, compared to birds sampled in other locations, suggesting that trade, movement of birds, and/or mixing birds of various origins in live-poultry markets likely contribute to the perpetuation of NDV at least in Nigeria. This is also reflected in the similarity of strains found in different Nigerian States. For instance sublineage 7b strains from 2009 from Sokoto and Yobe States were 99.4 to 99.6% similar to each other, strain chicken/Nigeria/VRD07-141/2007 from Sokoto [354] had 98.9 to 99.2% similarities with sublineage 8a strains from Yobe State (Figure 25). The presence of highly similar strains, especially those clustering in sublineage 8a but also in 9a, in different countries and the diversity of strains in each country suggest high exchange rates between regions. Selling of live birds is very common in West Africa and sometimes birds are carried over several hundred kilometres between their breeding and consumption location [339], indicating that movement of animals or mechanical transfer of infectious material would most likely contribute to the spread of the disease [153]. In Nigeria for instance, increased number of

NDV outbreaks have been reported during the dry season (November-March) which coincide with the high demand and trade of poultry for Christmas and New Year celebrations [355].

This would also suggest that these strains may have spread beyond the borders of the investigated countries. In Benin and Togo, 4.9% (119/2427) of the collected bird samples between end 2008 and 2010 were positive for NDV [266]. However, neither genetic nor pathogenicity information were provided and surveys in neighbouring countries would be crucial to confirm this hypothesis. Moreover, two lineage 9 strains were found in wild birds at a quarantine station in the USA. Although the origin of the birds could not be confirmed (N. Hines, pers. comm.), the fact that these strains have only been found in West Africa suggest that they were imported from the region.

In our study, NDV was more often detected in chickens, but viral RNA was also detected in the other investigated species, except in geese, probably due to the low number of samples available from this species. Ducks, turkeys and guinea fowls are considered generally more resistant to the disease than chickens, and ducks in particular may be infected while showing no symptoms. In 1997, velogenic NDV (sublineage 5b) was introduced in the UK and several flocks of chickens and turkeys were infected but clinical signs in turkeys were milder than in chickens, despite only minor genetic and antigenic differences of the viruses [202]. Differences in susceptibility to these strains between turkeys, ducks and chickens of the same age were confirmed by experimental inoculations [324; 356]. A striking difference was also observed between ducks and chickens inoculated with lineage 3e or 5d strains from China [357], where all chickens died within four days post infection for both strains while 0% and 10% mortality was observed in ducks infected with lineage 5d and 3e strains respectively. However, in Nigeria, ducks were significantly less often infected with NDV, compared to the other species, suggesting that they may not be an important player in the epidemiology of NDV in West and Central Africa. But when infected, ducks, turkeys and guinea fowl may act as apparently healthy carriers of virulent viruses and may contribute to their spread. The gathering of these species potentially sub-clinically infected in live-poultry markets, their rearing as free-roaming birds or in backyard farms increase their intermingling potential with chickens that are likely more susceptible.

In Nigeria, virulent strains were found in apparently healthy guinea fowls and ducks [238] and Servan de Almeida *et al.* [353] described the detection four virulent strains in apparently unvaccinated and healthy chickens and guinea fowl in Mali, questioning their virulence. Pathotyping results showed, however, that all strains tested had ICPI values between 1.7 and

1.87, characteristic of virulent strains [241]. In our study, virulent strains were also characterized in apparently healthy animals, but 43/45 infected apparently healthy animals were sampled in live bird markets where both vaccinated and unvaccinated animals can be found, and vaccination would reduce the expression of clinical signs [250].

It has long been accepted that the main factor of NDV pathogenicity is determined by the sequence of the F protein cleavage site. The presence of multiple basic amino acids between positions 112 and 116 together with a phenylalanine at position 117 leads to cleavage of the F_0 precursor in a wider range of cells resulting in systemic spread and increased pathogenicity [169]. Interestingly, two different F protein cleavage site sequences were found in sublineage 7b and 8a, and atypical ^{112}RRRKR*F^{117} sequences were observed, while the vast majority of virulent and avirulent share a glutamine at position 114 instead of an arginine. Only a few other strains belonging to lineage 4b, 4d, 5d, 5b and the four 3g strains from Madagascar also have Q114R or Q114K mutations. Reverse genetic experiments recently showed that a Q114R mutation reduced viral replication *in vitro* and *in vivo*, and attenuated the pathogenicity in day old chicks [358]. The additional I118V mutation – otherwise found in lineage 3c, in half of the lineage 6 strains and a few other exceptions – further decreased the pathogenicity. This could partially explain the apparently reduced virulence observed in the field, but is likely not only factor involved because both 114Q and 114R or 118I and 118V were found in healthy and sick animals in our study. The complete genome of these strains should be analysed, as the F protein is not the only virulence determinant, and experimental challenge would help to clarify the potential differences in pathogenicity.

On the other hand, Solomon et al. [359] mentioned "*increasing report of outbreaks of NDV in vaccinated flocks in Nigeria*", suggesting that the vaccine strains used may not efficiently protect the birds against challenge with the local strains. As NDV exist as a single serotype, it is commonly accepted that any vaccine strain should protect the birds from morbidity and mortality upon challenge with a virulent strain [250]. However, differences in reduction of viral shedding can be observed depending on the antigenic similarity between vaccine and challenge strains [360]. Except in some cases where variants viruses overcame the protection offered by vaccine strains, the commonly used vaccines still usually perform well. It was therefore suggested that other reasons such as suboptimal vaccination [361], inappropriate vaccination schedules or co-infection with immunosuppressive pathogens [248] may result in reduced immunity. In addition, poor vaccine conservation due to break in cold chain may also lead to suboptimal flock immunization.

In conclusion, the genetic information provided in this study showed that the virulent NDV strains enzootically circulating in West and Central Africa represent three new lineages of NDV. Although lineage 7, 8 and 9 strains are so far restricted to West and Central Africa, trade of domestic and exotic species may lead to further spread to neighbouring countries, as well as to other continents. Experimental inoculation of susceptible and vaccinated animals should be performed in order to investigate the antigenic similarity between field and vaccine strains, and assess the virulence of strains exhibiting atypical cleavage site of the fusion protein.

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Chapter 5:

Genetic diversity of Newcastle disease virus in wild birds and pigeons in West Africa

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Manuscript in preparation

1. Introduction

Newcastle disease virus (NDV), a Paramyxovirus, is one the most dangerous disease of poultry. Similarly to avian influenza, wild birds are thought to be reservoir of avirulent strains that could potentially be transmitted to poultry. NDV exhibit a broad range of pathogenicity for chickens and are divided into asymptomatic enteric, lentogenic, mesogenic and velogenic based on experimental inoculation of day-old chicks [166].

In West and Central Africa, several lineages of virulent strains are enzootic in poultry [241; 289]. The rather wide geographic spread of these strains in the region raised the question of the potential role of wild birds in their epidemiology. Several cases of lineage 4 were identified in doves and pigeons in Nigeria [289; 354] and South Africa [236]. In other wild bird species, detection of anti-NDV antibodies or NDV antigens were already reported in South Africa [285], Burkina Faso [362] and Nigeria [363; 364] but no genetic information was available. In this study, we investigated the presence of NDV in wild birds and pigeons in Nigeria and Côte d'Ivoire.

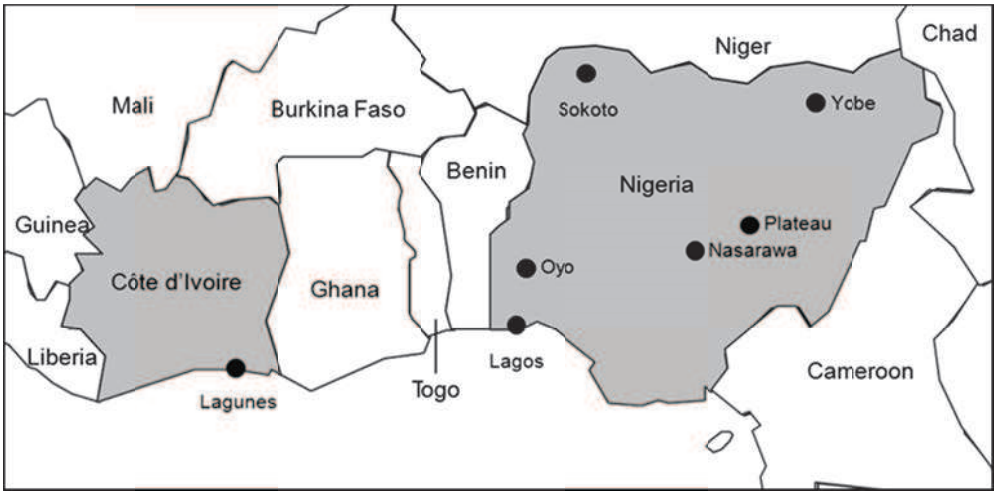

Figure 27. Collection sites in Nigeria and Côte d'Ivoire. Sampling provinces or states are indicated by black circles.

2. Material and Methods

2.1 Sample collection

A total of 1691 samples were collected in six Nigerian States (Oyo, *n*=798; Yobe, *n*=668; Plateau, *n*=154; Sokoto, *n*=37; Lagos, *n*=32; Nasarawa, *n*=2) between 2006 and 2011 during active surveillance (Table 14, Figure 27). Oropharyngeal and cloacal swabs as well as fresh

fecal samples were collected after ensuring the species of origin. Different organs from 19 birds were collected during passive surveillance in Lagunes province in Côte d'Ivoire between 2006 and 2008 (Table 14, Figure 27).

Table 14. List of sampled wild birds by Order in Nigeria and Côte d'Ivoire. The number of positive samples is indicated in brackets.

Order	No. family/order	No. species/order	No. samples (No. positives)	
			Nigeria	Côte d'Ivoire
Galliformes	1	2	1	1
Anseriformes	1	3	273 (5)	
Ciconiiformes	1	1	6	
Pelecaniformes	1	3	5	1
Accipitriformes	1	4	8	
Gruiformes	1	5	13	
Charadriiformes	5	6	80	
Columbiformes	1	9	274 (4)	11
Cuculiformes	1	1	2	
Coliiformes	1	1	9	
Coraciiformes	2	6	32	
Piciformes	3	8	26	
Passeriformes	28	92	896	5 (1)
unknown			66	1
Total	47	141	1691	19

2.2 Nucleic acid extraction, RT-PCR, and sequencing

All swab samples were homogenized and stored in 500 µl of virus transport medium [258]. RNA was extracted from 140 µl of medium using QIAamp Viral RNA Mini Kit (Qiagen, Venlo, The Netherlands) or from 50 µl using MagMAX^{TM}-96 AI/ND Viral RNA Isolation Kit (Life Technologies, Merelbeke, Belgium) with KingFisher (Thermo Fisher, Waltham, Massachusetts, USA). Organs were homogenized in 600 µl of lysis buffer of RNeasy Mini Kit (Qiagen) with stainless steel beads and TissueLyzer II (Qiagen). RNA extraction was then performed according to the manufacturers' instructions. NDV detection was performed by a multiplex real-time RT-PCR [365] on samples from wild birds from Nigeria. A nested PCR was used to detect NDV in pigeons from Nigeria and wild birds from Côte d'Ivoire [289; 329]. Partial or full length F genes were amplified using several overlapping fragments in (semi-) nested PCRs. PCR product purification and sequencing was performed as described before [289].

2.3 Data analysis

Sequences were assembled, analyzed and edited using SeqScape v2.5 (Applied Biosystems) and BioEdit [260]. Kimura distances were calculated according to the Kimura 2-parameter model on partial (375 nt) or complete (1662 nt) F gene sequences. Phylogenetic analyses were performed on 918 complete and 3303 partial F gene sequences using the Neighbour-Joining method, the Kimura 2-parameter model and 1000 bootstrap replicates as implemented in MEGA v5.03 software [261]. Representative strains were selected based on these preliminary analyses and are displayed in Figure 28 and Figure 29.

3. Results and Discussion

Ten out of 1691 samples tested positive for NDV. Full F gene sequencing was attempted in order to investigate their phylogenetic relationship. The complete sequence could not be obtained for one of the ten samples.

3.1 Lineage 1

Five highly similar sequences (Kimura distance 0 to 0.1%) from Spur-winged Geese (*Plectropterus gambensis*) sampled in the Dagona Wildlife Sanctuary in Yobe State during three consecutive days in April 2008 formed a cluster within lineage 1 (Figure 28 and Figure 29), suggesting that they share a very recent common ancestor. Their cleavage site sequence ^{112}GKQGR*L^{117} was typical of avirulent lineage 1 strains. All strains belonged to a cluster found mainly in migratory wild waterfowl and domestic ducks in Asia [235; 366-369], the USA [192] and Europe [370; 371]. They are not related to the use of live vaccine strains derived from Ulster or Queensland/V4, contrary to the other lineage 1 strains found in South Africa, Nigeria and Cameroon (Figure 29) [218; 289]. Spur-winged Geese intermingle with Eurasian migratory birds overwintering in the Dagona Wildlife Sanctuary [279], suggesting that the five avirulent lineage 1 strains may have been introduced by migratory birds. However, introduction by poultry cannot be ruled out, despite the ban on poultry product import in Nigeria since 2002. The lack of sequences from Africa cannot exclude the possibility that such lineage 1 strains also circulate in African wild birds.

Interestingly, 650 birds belonging to 44 other species were also sampled during the same period of time (March-April 2008) in the same region, but no other species tested positive. In the Dagona Wildlife Sanctuary, Spur-winged Geese and White-faced Whistling Ducks

(*Dendrocygna viduata*) were the only two species carrying avian influenza H5N2 viruses among 17 [272] and 44 [273] species investigated, suggesting that they may play a particular role in the epidemiology of both avian influenza and NDV in the region. These two species have similar behavior, *i.e.* they forage in shallow water, are both intra-continental migrants but they gather around waterbodies outside the breeding season [280] where they can intermix with migratory birds [279]. This may suggest that they were infected by contaminated water or contaminated feces usually present in high quantities at roosting sites, as asymptmatic enteric strains are preferentially shed by the digestive rather than the respiratory route.

In South Africa, 74% (34/46) of wild aquatic birds sampled in an intensive ostrich farming area were seropositive for NDV [285]. It was postulated that they may act as a reservoir for introduction of NDV in ostriches in which outbreaks often occur [236; 285]. Free-roaming ducks are reared in high numbers in the wetlands in Northeastern Nigeria [320] and often intermingle with wild waterfowls such as Spur-winged Geese, indicating that NDV viruses may sometimes be transmitted from wild to domestic Anatidae. Even though ducks are more resistant to infection with NDV, they can shed the virus for long period of time, could further spread the virus and also introduce it into chickens, where the virus could mutate and become more virulent, as has happened in Australia in 1998 [181].

3.2 Sublineage 4b

Four sequences from pigeons sampled in live bird markets clustered in sublineage 4b (Figure 28). Three very similar strains (Kimura distances 0.1 to 0.2%) were collected in Oyo State in 2007. The fourth sequence from a pigeon sampled in Lagos State in 2009 had a Kimura distance of 0.7 to 0.8% to the three strains from 2007. All strains had predicted virulent cleavage site sequence ^{112}RRKKR*F^{117}.

Phylogenetic analyses based on 375 nucleotides of the fusion gene revealed that these four strains clustered with two strains from Oyo (parrot/Nigeria/NIE139/2007) and Sokoto (pigeon/Nigeria/NIE95/2007) States (cluster 1, Figure 29) and this group was most closely related to pigeon isolates from Saudi Arabia from 1996 (AY175760). However they were not directly related to other pigeons strains from Jigawa State (cluster 2), nor with strains from pigeons, doves and chickens from South Africa (clusters 3 and 4, Figure 29).

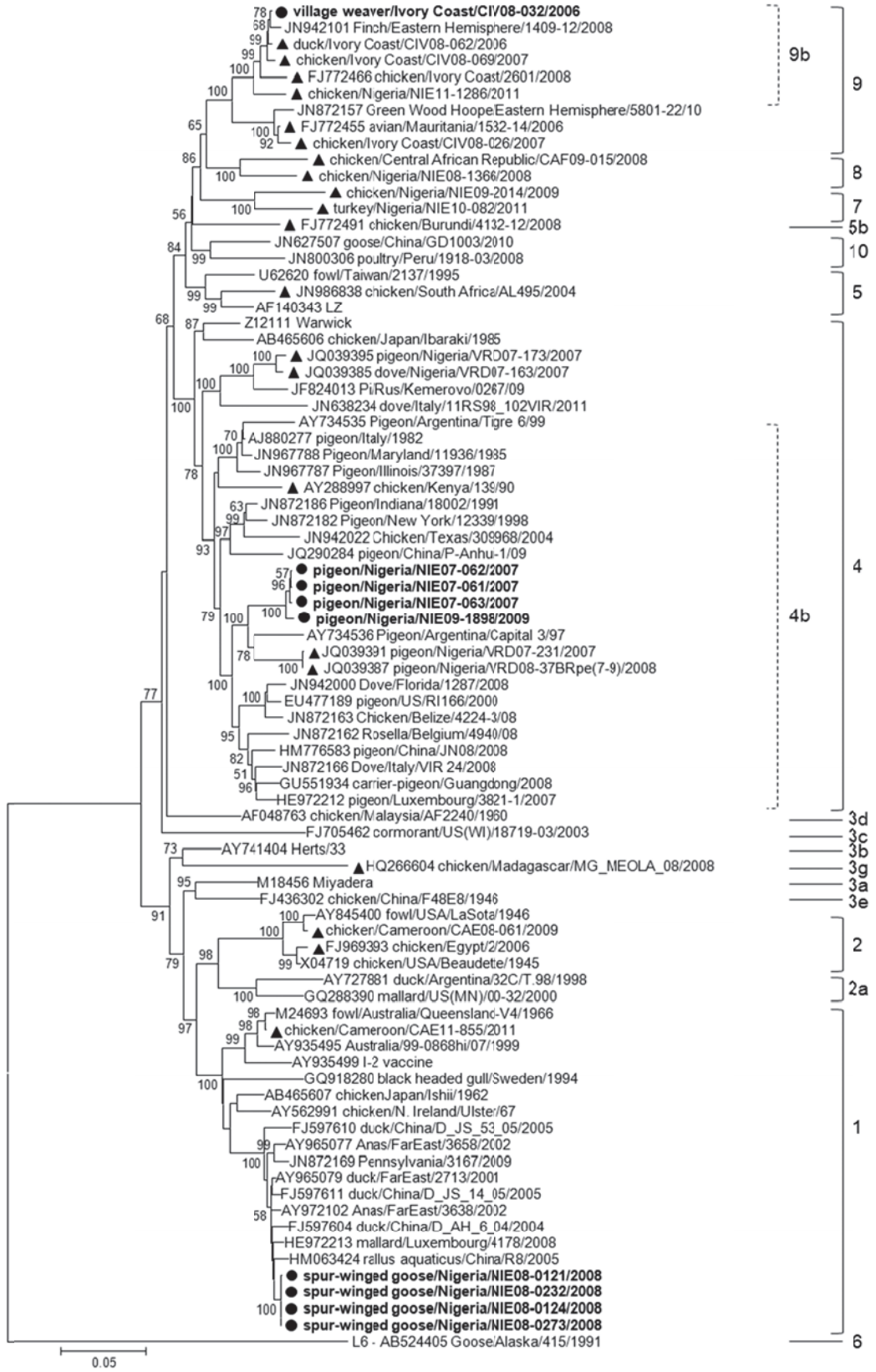

Figure 28. Phylogenetic analysis of complete F gene sequences (1662 nt). Sequences generated in this study are indicated by the symbols ●. Previously published sequences are indicated with their accession numbers and African strains are indicated by ▲. Only bootstrap values ≥50% are shown. The scale corresponds to number of base substitutions per site.

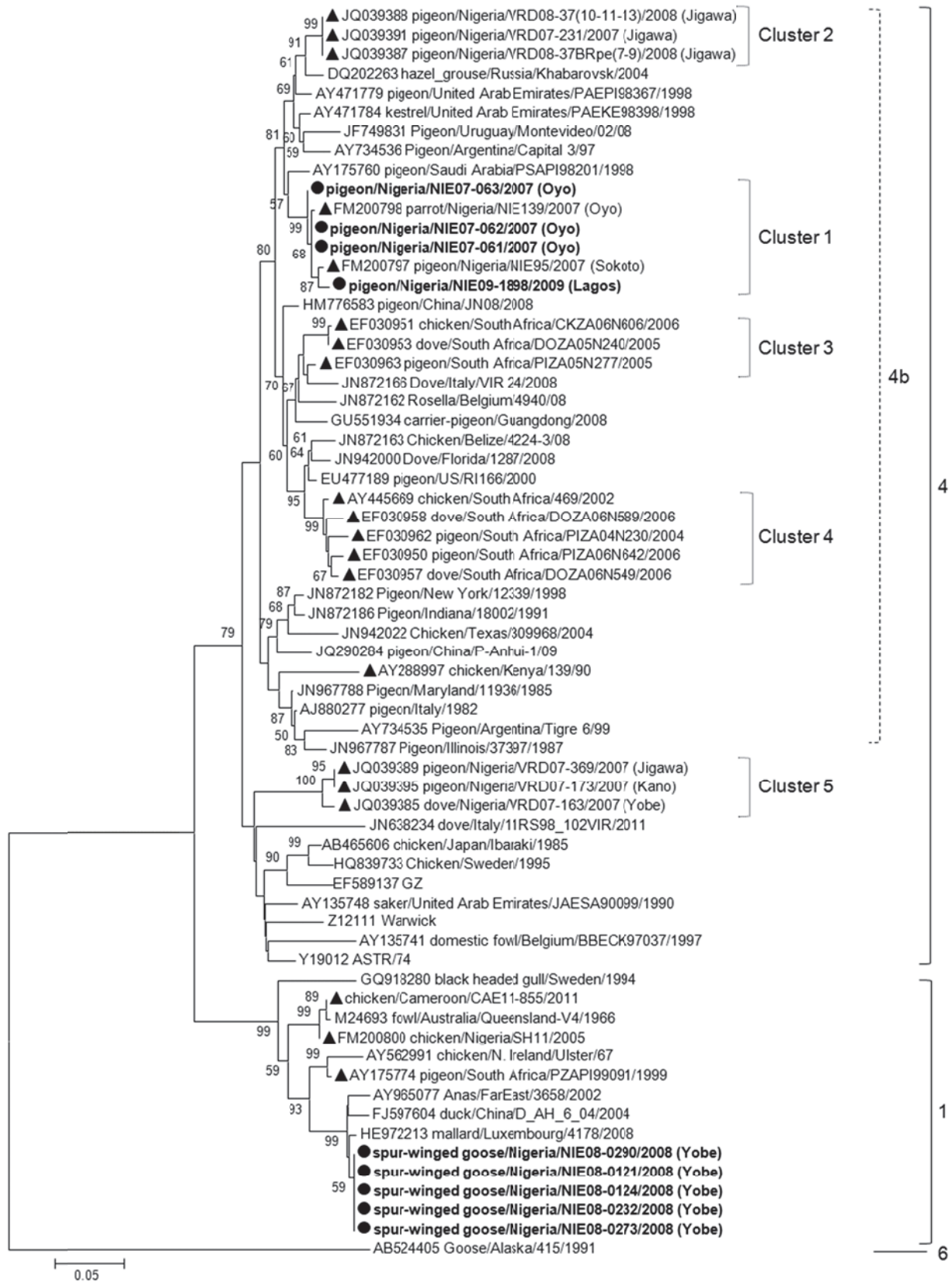

Figure 29. Phylogenetic analysis of partial F gene sequences (375 nucleotides). Sequences generated in this study are indicated by the symbols ●. Previously published sequences are indicated with their accession numbers and African strains are indicated by ▲. Only bootstrap values ≥50% are shown. The scale corresponds to number of base substitutions per site.

This suggests that several introductions of lineage 4, and in particular sublineage 4b, strains occurred in Africa during the last ten years. There seem to be a geographic repartition of sublineage 4b strains in Nigeria, with cluster 1 only found in South- and Northwestern Nigeria only, while cluster 2 is found only in Northeastern Nigeria. A geographic distinction was also recorded in South Africa where cluster 3 was found in the Northern part and cluster 4 in the Southern part of the country [236]. However, bird trade, including of pigeons that are often reared for meat in Nigeria [372], will likely result in virus dissemination, and geographic patterns may disappear in the future.

Sublineage 4b strains may also be transmitted to feral doves and lead to high mortality rates, as documented in other countries such as the US [195] or Italy [196]. Sublineage 4a (cluster 5, Figure 29) was also detected in sick pigeons and doves in Nigeria [354]. In Italy, mass mortality of Collared Doves (*Streptopelia decaocto*) was caused by viruses from this sublineage [222]. In our study, 169 birds from nine wild dove species were also sampled but none were positive for NDV, suggesting that sublineage 4b strains were not enzootic in wild doves in the region. However, passive surveillance in wild Columbidae species would be helpful to assess the risk posed by lineage 4 strains.

In addition to the damage to Columbidae, transmission of PPMV-1 to chickens can lead to serious outbreaks [228; 373]. Spread to chickens also occurred twice in South Africa (clusters 3 and 4, Figure 29) [236]. Although PPMV-1 was not found in more than 3000 domestic birds sampled in West and Central Africa (Chapter 4), the mingling of species in live-poultry markets increases the risk of transmission of PPMV-1 viruses to chickens, for which they may be virulent, at least after circulation and adaptation [174; 229]. Transmission of virulent PPMV-1 strains to chickens would further increase the viral pressure on the poultry industry in Africa, already weakened by the circulation of other virulent NDV strains [241; 289].

3.3 Lineage 9

One strain from a Village Weaver (*Ploceus cucullatus*) clustered together with strains from chickens and ducks from Côte d'Ivoire and Nigeria within sublineage 9b (Figure 28). Kimura distances ranged from 0.4% to chicken/Ivory Coast/CIV08-062/2006 to 3.2% to chicken/Nigeria/NIE11-1286/2011. Its cleavage site sequence ^{112}RRQKR*F^{117} was similar to the other lineage 9b isolates and predictive of a virulent strain. The virulent nature of the virus was also suggested by the death of the bird, although another cause of death was not

ruled out during a necropsy. This isolate represents so far the only case of a newly defined lineage 7, 8 or 9 strain in wild birds, except for two strains finch/Eastern Hemisphere/1409-12/2008 and Green Wood Hoopoe/Eastern Hemisphere/5801-22/10 isolated from a quarantine station in the U.S. during import testing (N. Hines, pers. comm.). Village Weavers live in groups in urban or rural areas, feed on insects and seeds and sometimes interact with free-roaming poultry while feeding. Thus Village Weavers were most probably infected by direct or indirect contact with sick poultry as other cases of sublineage 9b were found in chickens in the surrounding areas in the Region of Lagunes in Côte d'Ivoire (Chapter 5). Similarly, cases of spill back of virulent NDV strains to wild birds were already described in China, where House Sparrows (*Passer domesticus*) were infected with lineage 5 strains similar to those causing outbreaks in poultry in the same area [247]. The role of these bridge species, *i.e.* birds living in urbanized/rural area and having frequent contacts with domestic birds, in the epidemiology and spread of virulent NDV is unclear, as the virulence of viral strains may vary between species. However, in this particular case, the development of symptoms, followed by the death of the birds, likely limited the spread of the virus. So far, in wild birds, virulent strains seem only enzootic in cormorants in North America [200] and in pigeons worldwide [223; 225; 337], but monitoring of infection status of wild birds will be essential to better understand the host range of the virulent strains enzootic in West and Central Africa.

In our study, no lineage 6 strains were found in wild birds. Concern about lineage 6 strains has increased after some virulent viruses, derived from avirulent lineage 6 strains, were at the origin of outbreaks in Ireland in 1990 [180]. Their presence in Africa was never truly investigated although it could be expected. Lineage 6 strains were identified in migratory birds in the USA [192], and Europe, and could be introduced by them in wetlands of West Africa similarly to lineage 1 strains. Studies in live bird markets in the USA showed that similar lineage 6 strains were found in wild waterfowl or shorebirds and poultry present at live bird markets [192]. Ducks and chickens were also infected by lineage 6 strains in live poultry markets in China and Hong Kong, suggesting that interactions with wild birds may have resulted in virus transmission [235; 367]. Therefore, screening poultry in markets in Africa could help to investigate the epidemiology of lineage 6 in addition to wild bird sampling, which constitutes a harder task.

In conclusion, typical wild-type lineage 1 strains were found in wild waterfowl in wetlands in Northeastern Nigeria and may have been introduced in Africa by migratory birds.

Phylogenetic analyses also revealed that sublineage 4b strains were introduced on several separate occasions in Africa and may constitute a risk for poultry, while virulent sublineage 9b strain was found in a dead wild bird, likely resulting from a spill back from sick poultry.

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Chapter 6:

Characterization of Newcastle disease virus in Luxembourg, 2006-2011

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This study has been submitted to Applied and Environmental Microbiology.

1. Introduction

Newcastle disease, caused by virulent strains of Newcastle disease virus, is the most important viral disease of poultry together with highly pathogenic avian influenza. Newcastle disease virus (NDV), or avian paramyxovirus type-1, is a negative single-stranded RNA virus belonging to the *Avulavirus* genus in the *Paramyxoviridae* family. Based on clinical signs in chickens, several pathotypes have been defined [166]. The sequence of the fusion (F) protein cleavage site is considered a major virulence determinant and it appears that a minimum of three basic amino acids between residues 112 and 116, followed by a phenylalanine at residue 117 are required for virulence [166]. But other factors are also involved in the virulence phenotype as indicated by strains found in pigeons around the world and in healthy migratory ducks in Alaska that have a virulent cleavage site motif but are not always virulent for chicken by standard pathogenicity tests [171; 374; 375].

Besides its phenotypic heterogeneity, NDV is also genetically diverse and several phylogenetic (sub-)lineages are recognized but its diversity is still unfolding. Lineage 1, 6 and the newly described lineage 2a are mainly constituted by avirulent strains [186; 192]. Lineage 2 contains a broad spectrum of strains but nowadays, mainly avirulent strains are found. Lineage 3 to 5 contain almost exclusively viruses with a virulent cleavage site [186].

Wild birds constitute a natural reservoir of viruses of low virulence for chicken. In particular waterbirds may play an important role in NDV epidemiology due to shedding of viral particles into the aquatic environment more favorable for virus stability and their potential for long distance dissemination by migration [376]. Successions of outbreaks in the UK, Sweden, Denmark and Finland between 1996 and 2005 were all related at least on the basis of strain similarity but no direct epidemiological link was found, suggesting multiple introductions of viruses from the same pool by wild birds [202]. Moreover, isolation of similar viruses in wild birds (a goosander in Finland, a cormorant in Denmark) together with the proximity to water of a significant number of the affected flocks was suggestive of a wild bird reservoir at least of sublineage 5b strains in Western and Northern Europe [203]. Another more common threat for poultry arises from pigeon paramyxovirus type-1 (PPMV-1) strains, variants of NDV grouping in sublineage 4b. PPMV-1 strains exhibit a broad range of pathogenicity for poultry [196; 197; 374] and pathogenicity may increase after serial passages in chickens [173; 174; 196; 197; 229; 374]. On several occasions, PPMV-1 viruses were transmitted to chickens, leading to outbreaks [228; 373].

During the past decade, virulent viruses from sublineages 4a, 4b, 4d, 5b and 5d have been detected in wild and domestic birds in several European countries [203]. Avirulent lineage 1, 2 and 6 have also been reported sporadically. However, in Europe, only few sequences from wild birds are available, despite their importance for epidemiological surveillance. In this retrospective study, we examined stored samples collected in the framework of avian influenza surveillance in Luxembourg to investigate the presence of avirulent and virulent NDV in wild and domestic birds. All viruses found had a predicted low virulence, except for three PPMV-1 strains.

2. Material and Methods

2.1 Sample collection

Between January 2006 and July 2010, pooled tracheal and cloacal swabs (*n*=576), cloacal swabs (*n*=196), tracheal swabs (*n*=22) or fresh faeces (*n*=337) were collected during active and passive surveillance for avian influenza virus. The majority of the samples originated from wild birds (*n*= 1003), but domestic birds were also sampled, including chickens (*n*=120), turkey (*n*=1), quail (*n*=1), peafowl (*n*=1) and pheasants (*n*=2; Table 16). Samples from healthy passerines were mainly collected with mist nests during migration surveys at three locations: Nospelt, Ubersyren and Schifflange. Wild waterfowl, primarily targeted for avian influenza surveillance, were mainly sampled along the Moselle River in Remich and Wasserbillig. Some samples from injured or sick animals, especially from birds of prey, were also collected at a wildlife shelter while exotic species (*n*=3) were sampled at a zoological park and at the international airport. The other bird species were sampled throughout the country but with a bias towards the southern part of the country (Figure 30).

All swab samples were immediately placed in 500 µl of virus transport medium (PBS pH 7.0 with 2000 U penicillin/ml, 200 mg streptomycin/ml, 2000 U polymyxin B/ml, 250 mg gentamicin/ml, 60 mg ofloxacin/ml, 200 mg sulfamethoxazole/ml and 2.5 mg amphotericin B/ml) in the field. All samples were kept refrigerated until arrival at the laboratory. Approximately 100 mg of fecal samples were homogenized in 500 µl of virus transport medium upon arrival at the laboratory. 786 samples were processed immediately and cDNA was kept at -20°C, while 345 samples were stored at -80°C before being processed. The majority of the samples (86%, 968/1131) were stored at -80°C within 48h after collection.

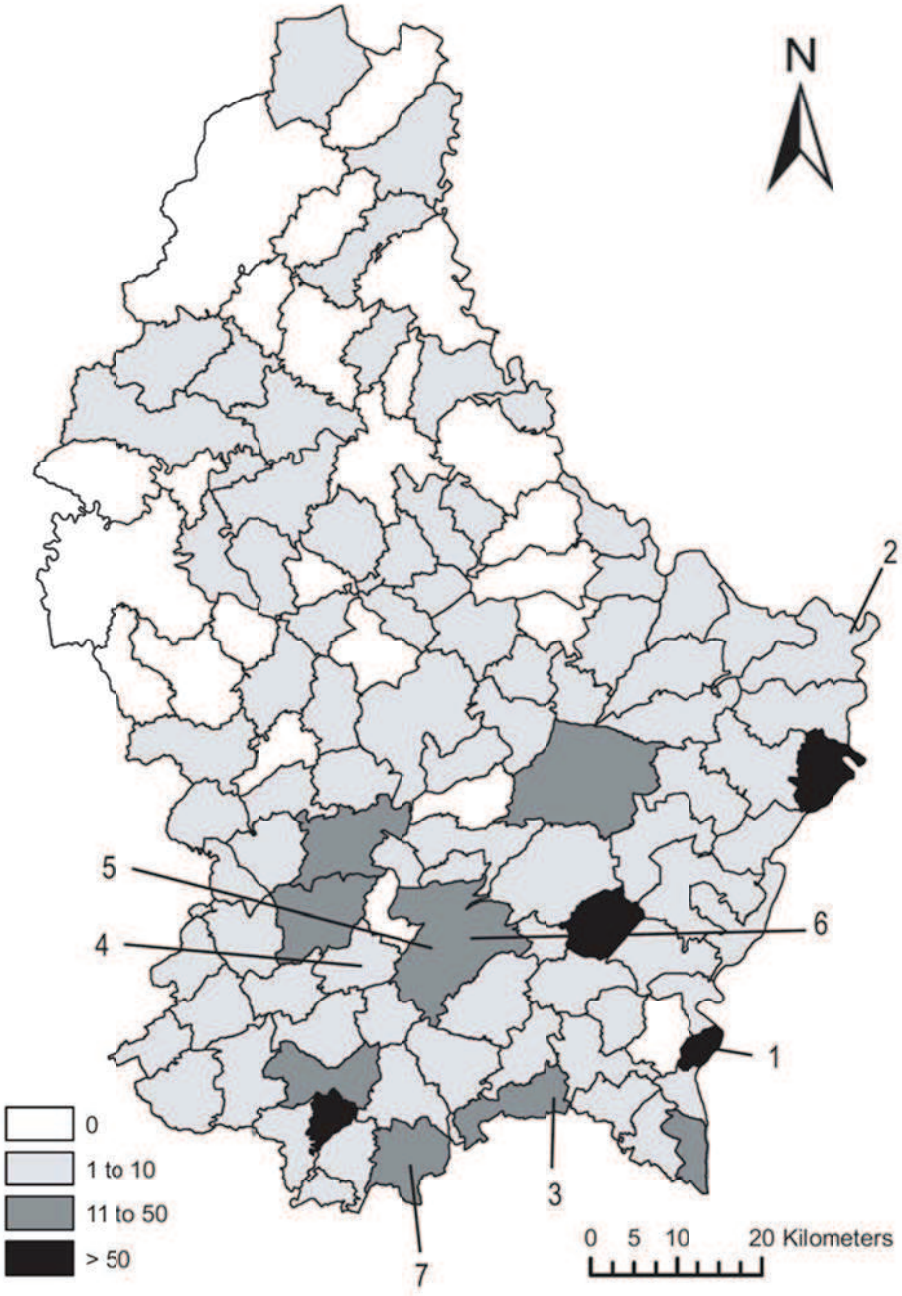

Figure 30. Geographic distribution of collected samples by municipalities in Luxembourg. The shading corresponds to the number of samples collected per municipalities. The numbers indicate the origin of the following isolates: 1) duck/Luxembourg/26/2006 and pigeon/Luxembourg/119/2006; 2) great cormorant/Luxembourg/2547/2006; 3) chicken/Luxembourg/2871-18/2007; 4) duck/Luxembourg/3785/2007 and duck/Luxembourg/3786/2007; 5) pigeon/Luxembourg/3821-1/2007; 6) mallard/Luxembourg/4178/2008. The strain pigeon/Luxembourg/2657-2/2006 originated from an animal rescued at the animal wildlife shelter in Dudelange (7).

2.2 Nucleic acid extraction, PCR, and sequencing

RNA was extracted from 140 µl of medium using QIAamp Viral RNA Mini Kit (Qiagen, Venlo, The Netherlands) or from 50 µl using MagMAX^{TM}-96 AI/ND Viral RNA Isolation Kit (Life Technologies, Merelbeke, Belgium) with KingFisher (Thermo Fisher, Waltham, Massachusetts, USA). Screening for lineage 6 was only performed on the 345 samples collected after June 2007 (according to [365]). Lineages 1 to 5 detection by nested PCR [289; 329] and sequencing of positive PCR products [289; 329] was carried out as described previously. Whenever enough material was available, the entire F gene was amplified using several primer combinations in (semi-)nested PCR formats (Table 15).

Table 15. Sequences of primers used to amplify and sequence partial or full F genes.

Primer	Orientation	Sequence (5' – 3')	Localization	Reference
FOP1	Forward	TACACCTCATCCCAGACAGGGTC	F gene	[329]
FOP2	Reverse	AGGCAGGGGAAGTGATTTGTGGC	F gene	[329]
FIP1	Forward	TACTTTGCTCACCCCCCTT	F gene	[329]
FIP2	Reverse	CATCTTCCCAACTGCCACT	F gene	[329]
M610	Forward	CTGTACAATCTTGCGCTCAATGTC	M gene	[218]
P1	Forward	ATGGGCYCCAGAYCTTCTAC	F gene	[208]
F581	Reverse	CTGCCACTGCTAGTTGTGATAATCC	F gene	[218]
F-4639f	Forward	TGAYGGCAGGCCTCTT	F gene	this study
F-4932f	Forward	CAACCGCTGCACAGATAA	F gene	this study
F-4954f	Forward	AGCTGCGGCYCTRATACAA	F gene	this study
F-5042f	Forward	GAGGTCACYGACGGATTAT	F gene	this study
F-5488f	Forward	TCAGCACTTGTCCCAAA	F gene	this study
F-1258-R	Reverse	ACATTGCATGAWTGTCTRTC	F gene	[241]
F-5566r	Reverse	CAGTATGAGGTGTCAAGTT	F gene	this study
F-5749f	Forward	AGACCCTCCAGGYATCA	F gene	this study
F-5888f	Forward	GGCTCAGTGGGGAAT	F gene	this study
F-6086f	Forward	GGTACACTTAGCCTGRTHTT	F gene	this study
F-6146r	Reverse	CTTYTGTTGCGCCTTT	F gene	this study
F-7979-R	Reverse	AGRGCCACYTGCTTRTATA	HN gene	[241]

2.3 Statistical analyses

Statistical analyses to assess if differences in sampling or sample processing had an effect on the outcome of the detection tests were performed using the Chi-square test with Yates correction in SigmaPlot software.

Table 16. List of sampled domestic and wild bird species in Luxembourg between 2006 and 2010 tested for lineages 1 to 5 and lineage 6 and lineage (L) classification of positive samples.

Family	No. positives/ No. tested for lineages 1 to 5	No. positives/ No. tested for lineage 6	Yearly distribution of positive samples		
			2006	2007	2008
Phasianidae [a]	1/125	0/62		1x L2	
Anatidae	4/340	0/92	1x L2	2x L1	1x L1
Phalacrocoracidae	1/3	0/1	1x L2		
Ardeidae	0/13	0/2			
Accipitridae	0/135	0/7			
Falconidae	0/26	0/6			
Rallidae	0/52	0/4			
Laridae	0/8	-			
Columbidae	3/59	0/11	2x L4b	1x L4b	
Psittacidae [b]	0/3	0/3			
Strigidae	0/12	0/1			
Tytonidae	0/14	0/1			
Picidae	0/5	0/1			
Corvidae	0/20	0/3			
Paridae	0/5	0/2			
Hirundinidae	0/9	0/9			
Phylloscopidae	0/5	0/2			
Acrocephalidae	0/42	0/32			
Locustellidae	0/4	0/3			
Sylviidae	0/95	0/37			
Sturnidae	0/7	-			
Turdidae	0/21	0/15			
Muscicapidae	0/9	0/4			
Passeridae	0/42	-			
Prunellidae	0/2	0/1			
Motacillidae	0/4	-			
Fringillidae	0/1	0/1			
Emberizidae	0/4	0/3			
Undetermined	0/66	0/42			
Total	9/1131	0/345	4/619	4/349	1/84

[a] domestic species including chicken, turkey, quail, peafowl and pheasant; [b] exotic species

2.4 Sequence analyses

Kimura distances were calculated according to the Kimura 2-parameter model on partial (240 nt) or complete (1662 nt) F gene sequences. The sequence lengths used for distance calculations are mentioned between brackets in the Results section. Phylogenetic relationships were inferred by comparing the Luxembourgish strains with all NDV sequences available on NCBI (downloaded in June 2012) after dataset curation. Datasets were aligned using ClustalW [291]. Trees were calculated with the Neighbour-Joining method, using the Kimura 2-parameter model and 1000 bootstrap replicates as implemented in MEGA v5.03

software [261]. Representative strains were selected based on these preliminary analyses and are displayed in Figure 31 and Figure 32. The classification nomenclature was used according to Aldous *et al.* [186] and the nucleotide numbering of F gene sequence according to Kho *et al.* [329]. Sequences were submitted to GenBank under accession numbers HE972209 to HE972217. The following strain nomenclature was used: host/country/strain number/year.

3. Results

None of the 345 samples tested for lineage 6 was positive for this lineage. A total of 9 samples out of 1131 were positive in the PCR detecting lineages 1 to 5 NDV strains (Table 16), corresponding to an overall prevalence of 0.8% during the 2006-2010 period (4/619 in 2006, 4/349 in 2007, 1/84 in 2008, 0/67 in 2009 and 0/12 in 2010). Statistical analyses revealed that the type of samples, the time between collection and storage (categorized as less or more than 48h) or the type of material used in the PCR (cDNA stored at -20°C or freshly prepared from original samples stored at -80°C) had no significant effect on the detection of NDV (*p*-values=0.816, 0.847, 0.583 respectively). However, higher prevalence might have been found if virus isolation or a more sensitive RT-PCR protocol had been used. The apparent absence of NDV in 2009-2010 as well as in the northern part of the country (Figure 30) was most probably due to a suboptimal surveillance effort rather than a disappearance of NDV in Luxembourg or the existence of regional differences.

Phylogenetic analyses of partial (9 strains; Figure 31) and complete (5 strains; Figure 32) F gene sequences revealed an equal distribution of the samples in 3 genetic lineages.

Lineage 1. Three similar strains (Kimura distances from 0 to 0.5%, 1662 nt) from ducks, including one Mallard *Anas platyrhynchos*, clustered in lineage 1. They were clustering together with strains from waterfowls from Finland, the Far East and China (Figure 31 and Figure 32) and were most closely related to each other based on complete F gene sequences (Figure 32). They all add a Kimura distance of 0.4% (240 nt) to mallard/Finland/9360/2010. The deduced amino acid sequence of the F protein cleavage site ^{112}GKQGR*L^{117} was typical of avirulent lineage 1 strains.

Figure 31. Phylogenetic analysis of partial F gene sequences based on nucleotides 332–571. Sequences generated in this study are indicated by the symbols ● (strains presented in Figure 31 and Figure 32) and ■ (strains presented in Fig. 1 only). Previously published sequences are indicated with their accession numbers. Only bootstrap values ≥ 50% are shown. The scale corresponds to number of base substitutions per site.

Lineage 2. Three samples from a chicken, a Great Cormorant *Phalocrocorax carbo* and a duck clustered in lineage 2, together with the commonly used vaccines LaSota and B1 (Figure 31). Kimura distances (240 nt) to the LaSota vaccine ranged from 0 (duck/Luxembourg/26/06) to 1.3% (chicken/Luxembourg/2871-18/07). Both duck/Luxembourg/26/06 and Great Cormorant/Luxembourg/2547/2006 exhibited a cleavage site typical of avirulent lineage 2 strains, ^{112}GRQGR*L^{117}, while the chicken/Luxembourg/2871-18/07 strain encoded ^{112}GGQGR*L^{117} due to a non-synonymous A to G substitution at nucleotide position 380.

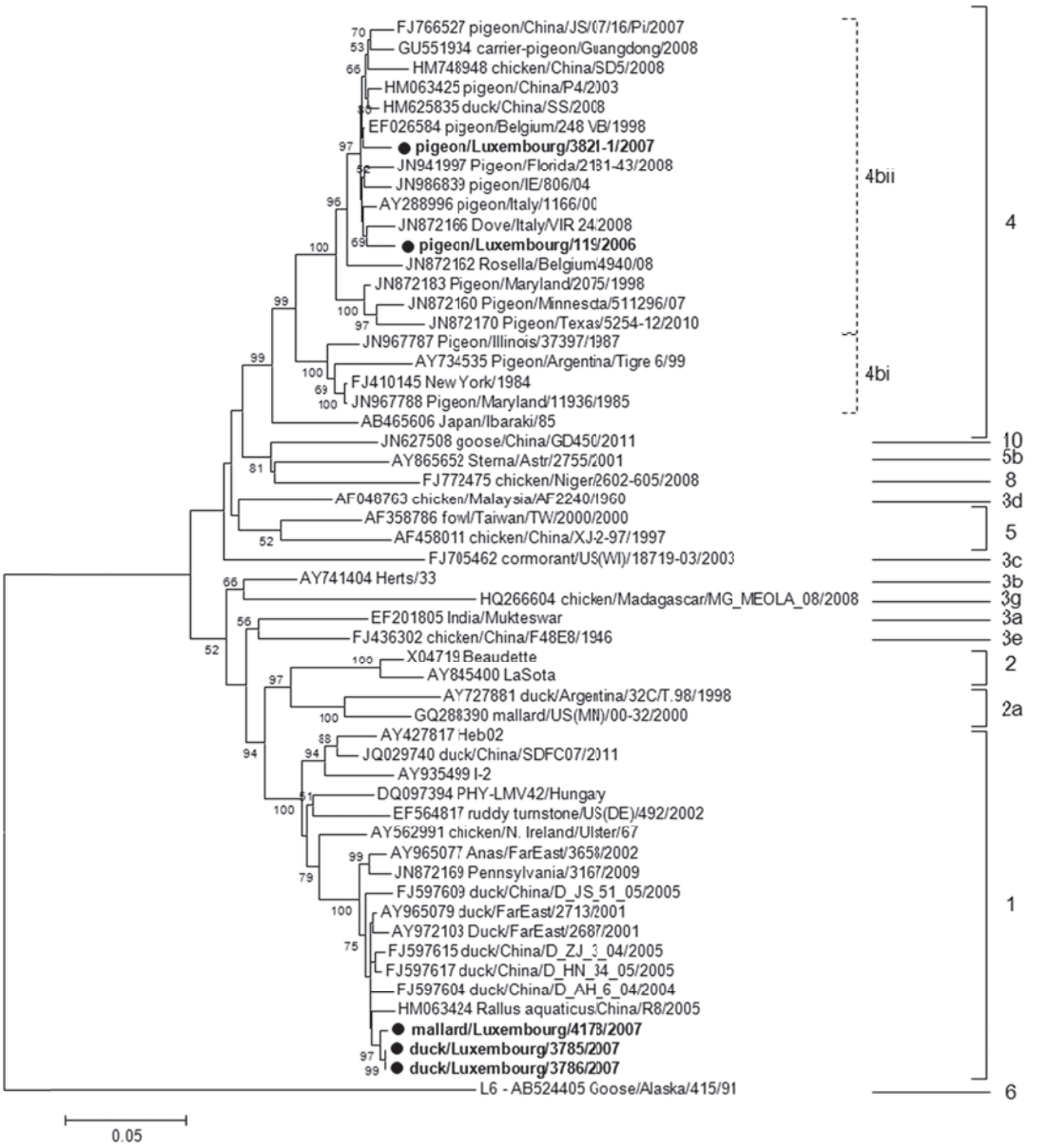

Figure 32. Phylogenetic analysis of complete F gene sequences (1662 nt). Symbols are as in Figure 31. Only bootstrap values ≥50% are shown. The scale corresponds to number of base substitutions per site.

Lineage 4. The last three sequences from pigeons *Columba livia var. domestica* clustered in sublineage 4bii (Figure 31), a subdivision of sublineage 4b including recent isolates mainly found in Columbiformes worldwide. Kimura distances ranged from 0.4% between pigeon/Luxembourg/2657-2/06 and pigeon/Luxembourg/119/06 (240 nt) to 2.6% between pigeon/Luxembourg/119/06 and pigeon/Luxembourg/3821-1/07 (1662 nt). All sublineage 4b strains encoded for virulent fusion cleavage site motifs ^{112}RRQKR*F^{117}, as defined by World Organization for Animal Health standard [166], and were similar to those of other previously described PPMV-1 strains.

4. Discussion

There is increasing evidence that wild waterbirds are natural carriers of avirulent lineage 1, 2a and 6 strains [192; 194; 370; 371; 377; 378]. It was thus not surprising to find five out of nine positive samples in waterbirds, including three avirulent lineage 1 strains in ducks in our study. Based on the full F gene sequences (Figure 32), these lineage 1 strains formed a monophyletic cluster, which may suggest that they evolved from a recent common ancestor and resulted from a single introduction event in Luxembourg. In this scenario, the detection of similar strains detected in August 2007 and October 2008 would indicate that avirulent viruses could be maintained in the local bird population throughout the year. However, the relationship between NDV isolates from Luxembourg and Finland based on partial F gene sequences was not clear (Figure 31). Complete F gene sequences from Finland, and from other European countries in general, would be needed to know whether the strains found in Luxembourg resulted from a single or two separate introduction event.

Lineage 1 strains were recently identified in migratory species in Finland [370]. Eurasian Teals, Mallards and Northern Shoveler migrate with variable movements depending on the weather and on the subpopulations. These three species, as well as other migratory Anatidae, are also commonly observed in Luxembourg during migration. In our study, the two ducks sampled in August were likely resident birds, but the Mallard sampled in October might have been migratory, although some may be resident in Luxembourg too. It is therefore possible that lineage 1 strains have been introduced by migratory species in one or two occasions.

Strains of sublineage 4b found in three Luxembourg pigeons likely originate from separate introduction events, as they do not share a direct common ancestor and intermingle with strains found in other countries. While PPMV-1 strains initially circulated mainly in racing

and show pigeons, they are now considered enzootic in feral pigeons and doves in countries such as Germany and Italy [196; 197]. Cases in pigeons have been detected almost every year between 2000 and 2009 in the neighbouring countries [203] and dissemination to Luxembourg is not unexpected.

While lineage 1, 2a and 6 strains are often detected in waterfowl, LaSota-like strains (lineage 2) are mainly found in poultry and are usually associated with the use of live vaccines [247; 289; 379]. In this respect, our finding of a vaccine-like strain in a Great Cormorant and a duck in Luxembourg is somewhat unusual. The detection of vaccine strains in non-vaccinated flocks suggested that lentogenic vaccine strains can spread at least within poultry [246]. In addition, wild type virus transmission between wild and domestic birds were already suspected to be at the origin of the similarity of strains found in wild birds and domestic birds in live bird markets [192; 380] or in flocks with possible contacts with wild birds [376], and of the spill-back of virulent strains into wild birds [247; 381]. Therefore it seems reasonable to expect that vaccine strains may also be exchanged between poultry and wild birds. Similar cases of strains close to LaSota or B1 vaccines reported in wild birds have been reported elsewhere, such as in Asia (China, India and Malaysia), Argentina or France (Figure 31). Although NDV vaccination is not allowed in Luxembourg, vaccinated animals are also sometimes imported (Losch S., pers comm.). Also bridge species such as sparrows that live in close proximity with domestic birds [247], in particular in regions where backyard chickens are commonly reared, or food/water contamination [228; 382] may have contributed to the transmission of LaSota-like strains to waterbirds in Luxembourg. On the other hand, wild birds may have been infected elsewhere in European countries where vaccination is sometimes allowed. Despite that the Great Cormorant infected with a lineage 2 strain was sampled in April, it is difficult to known whether this bird spent the winter locally, or was sampled during its northwards migration because Great Cormorants tend to migrate late in the season. Unfortunately, the other bird infected with a lineage 2 strain was not identified to the species level.

The only strain found in a domestic bird belonged to lineage 2, but did not seem to be directly related to a vaccine strain because of three mutations in a short region of the F gene leading to two amino acid substitutions, one being in the cleavage site. To our knowledge, this particular cleavage site sequence has not been reported before, but this strain is probably not virulent for chickens as it contains only one basic amino acid between residues 112 and 116

and a leucine at residue 117. Unfortunately no further information about potential clinical signs in the flock was available.

No virulent NDV strains were found in wild or domestic birds in Luxembourg, except for the three PPMV-1 strains. Although the latter are normally found in pigeons, PPMV-1 transmission to poultry was reported on a few occasions in Europe during the past decade [203]. Even if most cases occurred in small backyard flocks with low biosecurity, the circulation of PPMV-1 in pigeons represents a potential threat for the poultry industry. Also, the presence of avirulent strains in wild birds may be a risk for poultry. Although excessively rare in the field, virulent strains may develop from low virulent ones after mutation as was postulated for outbreaks that occurred in Ireland [180] and Australia [181]. This was also demonstrated by serial experimental passages in chickens [183], which may have given minor populations of virulent NDV in field isolates a selective advantage [383]. All these scenarios highlight the importance of virological surveillance and preventive measures to reduce the intermingling of wild and domestic birds.

In conclusion, we found avirulent lineage 1 strains in waterfowls in Luxembourg similar to those circulating in wild migratory birds in Finland, suggesting that these viruses represent typical avirulent strains found in European wild birds and that migratory birds may contribute to their spread. Detection of vaccine-like strains in wild waterbirds suggests the spread of vaccine strains, despite the non-vaccination policy in Luxembourg. Although the three PPMV-1 strains from pigeons were the only virulent strains found in Luxembourg, the presence of NDV in wild and free-ranging domestic birds justifies the need for continuous surveillance in wild and domestic birds.

5. Acknowledgments

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General discussion

The spread of HPAI H5N1 viruses that reemerged in 2003 in Asia after 5 years of unapparent circulation led to the biggest HPAI epizootic ever recorded to date. In addition, the incredible economic loss induced by stamping out, vaccination campaigns, movement and trade restrictions, this virus was the first HPAI virus to infect so many humans, resulting in more than 350 deaths in 15 countries. In addition to a high mortality rate of nearly 60%, scientists fear that HPAI H5N1 could reassort with seasonal human influenza viruses and acquire the capacity of human-to-human transmission that it still lacks. Although this has not happened in 15 years of circulation, some viruses definitely acquired mutations towards enhanced virulence or replication in mammals.

One event believed to have considerably contributed to the spread of HPAI H5N1 beyond Southeast Asia was the outbreak that happened in 2005 at the Qinghai Lake in western China, a major breeding site for migratory birds. Soon after that, HPAI H5N1 cases were recorded in Russia, the Middle East and Eastern Europe. By February 2006, HPAI H5N1 had reached Western Europe and Africa. Nigeria was the first African country to report HPAI H5N1 in February, but soon after, other African countries were also affected.

Phylogenetic analyses performed on viruses from different Nigerian states showed that three different viruses of clade 2.2 were introduced into the country in 2006, namely sublineages A, B (corresponding to clade 2.2.1 later defined by the WHO/OIE/FAO H5N1 Evolution Working Group) and C [123; 124]. Co-circulation of sublineages A and C eventually resulted in the emergence of $AC_{HA/NS}$ reassortants probably as early as in 2006. In **Chapter 1**, we have analyzed avian samples from Nigeria and Cameroon between 2006 and 2011. Influenza A viruses were only detected in Nigeria in 2006 and 2007. No positive sample was found in 2008 despite low levels of circulation as denoted by recorded outbreaks in Anambra state in February 2008 [114] and in Kano and Katsina states in June 2008 [265]. In July, surveillance in live bird markets in Gombe, Kebbi, Kano and Katsina states also allowed the detection of viruses more closely related to those circulating in wild birds in Western and Eastern Europe in 2007 rather than to African viruses, suggesting that they were more recently introduced [127; 257; 265]. The circulation of these viruses seemed restricted to Northern Nigeria, but our surveillance in Sokoto (North-West), Yobe (North-East), Plateau and Nassarawa (North-Central) states in 2008 did not show their presence.

Sequencing and phylogenetic analyses (**Chapter 1**) confirmed the predominance of sublineage A in 2006, at least in South-Western states. We did not find any sublineage C strains although not all positive samples were sequenced and we cannot exclude that some would contain sublineage C strains. Sublineage C has only rarely been detected in Nigeria [131; 384] despite being responsible for the first reported outbreaks in Nigeria. They also constituted the majority of the strains detected in the other sub-Saharan countries and all reassortant strains included one, two or four genes of sublineage C [128-130], suggesting that they must have co-circulated together with sublineage A. Similarly, sublineage B (clade 2.2.1) was found only in Lagos, Ogun and Oyo states between January and March 2006 [122; 124; 257] whereas it has been circulating uninterruptedly in Egypt since 2006. To date, no explanation was found as to why some sublineages seemed to have thrived in Nigeria while others disappeared quite quickly.

As suggested by the diversity of HPAI H5N1 strains found in a single live bird market in Oyo state (**Chapter 1**), movement of animals and trade likely played a great role in spreading H5N1 within Nigeria. Seck *et al.* also claimed that live-bird market along the borders appeared to have significantly contributed to the introduction of HPAI H5N1 into Niger, Burkina Faso and Côte d'Ivoire [385]. Within the Nigerian borders, risk factors analyses based on questionnaires filled in by farmers with our without HPAI H5N1 outbreaks in their farms, identified biosecurity measures, proximity to other farms, entry of external people in the farm and purchase of live poultry products as increasing the risk of HPAI H5N1 virus infection [264; 386].

As shown by the absence of HPAI H5N1 detection in 2008, 2009 and 2011 (**Chapter 1**) and the absence of outbreak reports to the OIE, the control measures taken by the local authorities seemed successful in containing the virus, contrary to Egypt. In affected countries, measures included active surveillance, stamping-out of sick and in-contact animals, disinfection of infected farms or villages, compensatory payments, restricted movement of birds and improved farmer awareness on biosecurity measures. Vaccination was implemented in Côte d'Ivoire, Sudan and Egypt and unofficially in certain parts of Nigeria [385]. However, some authors have questioned the true efficiency of control measures in containing completely HPAI H5N1 virus in West and Central Africa due to organisational problems [385; 387] and suggested that other factors, such as climatic conditions, less birds and less species intermixing in live-bird markets, lower animal densities, especially ducks, compared to

South-East Asia and Egypt, might have contributed to the self-extinction of the virus in the region [266].

Although it is clear that human activities have contributed to the spread of HPAI H5N1 once introduced in West Africa, its route of introduction in 2006 keeps the debate going and trade and migratory birds have been incriminated. In July 2005, the trajectory and timeline of the westward expansion of HPAI H5N1 seemed consistent with transport by the main routes linking China to Russia, *i.e.* the trans-Siberian railways [388] but dead migratory birds were also reported near the first reported outbreaks in Russia and Kazakhstan [114]. Outbreaks were reported in wild birds and poultry in Romania and Turkey in October while HPAI H5N1 is detected in migratory swans in Croatia [114]. In early 2006 however, reports of sick or dead wild birds, especially mute swans, arose from several European countries (including Greece, Italy, Slovenia, Germany, France, Austria, Bosnia-Herzegovina, Slovakia, Poland, Denmark, Sweden, Czech Republic, UK, Spain and Hungary) where no outbreaks in poultry was recorded at the time and were associated with unusual wild bird movement due to adverse weather in wintering areas around the Black Sea [389; 390].

However, HPAI H5N1 was first reported in Africa in February 2006, in the middle of the wintering season of migratory birds, thus inconsistent with the hypothesis of a recent introduction by migratory birds. The first reported outbreaks also occurred in a large commercial flock. Yet, it is possible that the virus was introduced before that and circulated unnoticed for some time, as suggested by noticed but unreported outbreaks in December 2005 [387]. A retrospective field survey also mentioned widespread mortality in local chickens in Jigawa state at the end of 2005. It is plausible that the clinical signs were not directly recognized as induced by HPAI viruses and confused with virulent NDV [320; 391]. Nevertheless, HPAI H5N1 virus was not isolated from wild birds in Africa, but for a few exceptions, none of which were long distance migrants [66; 286; 392]. Besides, the Nigerian government acknowledged that illegal poultry import still occurred in 2005 despite the ban imposed on poultry product import [393]. Valuable arguments in favour of each hypothesis on the introduction route of HPAI H5N1 in 2006 have been raised and this mystery may never be solved. The introduction of a new HPAI H5N1 virus in Northern Nigeria again fanned the flames. The virus still belonged to the 2.2 clade but was more closely related to strains circulating in wild and domestic birds in 2007 in Europe [127].

In order to better understand the potential exchanges of viruses between Eurasian and Africa, we collected samples from wild and domestic birds in the Hadejia-Nguru wetlands that are

extremely important for breeding and wintering waterfowl in Nigeria (**Chapter 2**; [279]). We did not detect any HPAI H5N1 viruses but found three LPAI H5N2 viruses in Spur-winged Geese (*Plectropterus gambensis*). The AIV prevalence in wild birds may seem relatively low (0.5%, 3/651) compared to 3.5% (159/4553) of positive waterbirds in African wetlands in early 2006 [274] and 2.9 % (70/2399) from January to March 2006, 2007, 2008 and 2009 [394] but falls in the range reported in Zambia (0.4%; 12/3094; [271]). However, the comparison of the origins of the birds sampled showed that Eurasian ducks had a 2.4 times [274] and 1.3 times [394] higher prevalence compared to Afro-tropical ones. In our study, no Eurasian ducks were sampled because the study was carried out from end March to end April and the majority of Eurasian migrants had already left. In addition, other factors such as the year and the location influence the prevalence of AIV in wild birds in Europe and North America, and it seems therefore reasonable to assume that differences are to be expected in Africa too, as shown by a two year study in five wetlands in Southern Africa [395].

In a continent-scale study, Gaidet *et al.* have examined several factors that could explain the AIV prevalence variations in Africa [396]. In temperate zones, it is believed that AIV may persist for prolonged periods of time in the environment and could serve as an infectious source during the next breeding season [9]. Peaks in prevalence in autumn have been explained by the massive congregation of juvenile and mature birds prior to and during migration. However, the virus will likely have a reduced persistence in the African environment due to higher temperatures and no massive synchronic bird movements happening. In Africa, higher prevalence rates were positively related to the higher densities of wild waterfowl [396], which in fact coincides with the arrival of Eurasian migrants and the congregation of African waterfowl in permanent waterbodies during the dry season as the water level and wetlands surfaces decrease. Given the higher influenza prevalence in autumn in Europe, it seems logical to assume that the Eurasian migrants may introduce viruses on their wintering locations, as also suggested by the origin of seven of the genes of the LPAI H5N2 viruses found in the Nigerian wetlands (**Chapter 2**) and the higher AIV prevalence in Eurasian compared to African ducks [274; 394]. Gaidet *et al.* also suggested that direct transmission by the respiratory route or by freshly shed infected faeces, promoted by higher bird densities, may play a predominant role in AIV transmission in Africa [396]. In addition, the prolonged breeding period of Afro-tropical birds may reduce the proportion of juvenile and naïve bird population at a given time, and could partially explain the lower prevalence rates recorded and the low but potentially continuous viral circulation [396].

Phylogenetic analyses performed on the eight genes of the three LPAI H5N2 viruses (**Chapter 2**) revealed that all but NS gene, were more closely related to genes circulating in the Eurasian wild bird influenza gene pool, especially in Western Europe. The three LPAI H5N2 viruses were always more closely related to each others, suggesting that they resulted from a single introduction event, and the time to the most recent common ancestor (TMRCA) calculated for each gene suggested that they were introduced between December 2006 and October 2007 (June 2005 to March 2008, 95% HPD). The Hadejia-Nguru wetlands are connected to Eurasia by three main migratory flyways, and migratory birds likely introduced those genes in Africa. Phylogenetic analyses on the NS gene, however, showed a different origin. Indeed, the NS genes of the Nigerian LPAI H5N2 viruses formed a sister clade to NS genes from South Africa and Zambia and the TMRCA of this African cluster ranged from November 1997 to March 2003 (95% HPD). This suggested that the LPAI H5N2 viruses resulted from a reassortant event including genes recently introduced from Eurasia, most probably Western Europe, and genes from a pool present in sub-Saharan Africa. Phylogenetic analyses on all African AIV sequences available (Figure 19, **Chapter 2**) highlighted the gene reassortments and suggested that some influenza viruses may be maintained in the wild bird population in Africa. Our hypothesis was corroborated by a recent study performed in Zambia that also showed the similarity of the majority of the genes of the viruses sequenced from ducks, geese and pelicans to those from South Africa but in various combinations [271].

Clearly, the introduction of HPAI H5N1 has benefited the research on influenza in Africa, but also to a certain extent, the research on NDV, as the same samples can be used to detect both pathogens. Virulent NDV are known to circulate for decades in Africa and are also considered as the major viral burden of the local poultry industry [339], but there was not much information about the genetic make-up of the viruses circulating in West Africa before the first results of this work. In **Chapter 3**, we provided the first (partial) sequences of NDV in West Africa. Phylogenetic analyses revealed that vaccine-like strains were found in commercial farms while virulent strains were mostly detected in backyard farms and live birds markets. We have also shown that the virulent strains present in Nigeria, Niger and Burkina Faso were diverse and seemed unrelated to other sublineage 5 strains (5a to 5e) but the genetic information was not sufficient to correctly assess the diversity and to reliably assure that they belonged to a new lineage.

In a follow-up study (**Chapter 4**), we expanded our research to three new countries, *i.e.* Côte d'Ivoire, Cameroon and Central African Republic, and increased the genetic information by

sequencing the complete fusion gene sequences, which allowed us to better characterize the extent of diversity and spread of these viruses. By comparing the mean genetic diversity within and between lineages to the groups of strains obtained in West and Central Africa, we defined three new lineages, namely lineages 7, 8 and 9, and seven sublineages, rather than three sublineages 5 as defined in **Chapter 3**. Except for sublineage 8b which was restricted to Central African Republic and sublineage 7b to Nigeria, the other four sublineages were found in at least two countries. The most spread sublineage was 8a found in Nigeria, Niger, Cameroon, Burkina Faso and Côte d'Ivoire (**Chapter 4**, [241]). The similarity of strains found in live bird markets and backyard farms (sublineage 7b and 8a), in several Nigerian states (sublineage 7b and 8a) or several countries (especially sublineage 8a) suggests that the viruses may be spread by poultry trade or movement of infected material, similarly to HPAI H5N1.

Similar patterns of disease incidence were observed between reported cases of NDV and the epizootic of HPAI H5N1 in 2006 and 2007 in Nigeria. The majority of HPAI H5N1 cases were reported between December and March [265] compared to November and January for NDV [397; 398], and a smaller peak was observed between May and August [265; 397]. It was proposed that increased movements of birds (trade and gifts) at the end of the year, due to Christmas and New Year celebrations, would likely contribute to the spread of both viruses [265; 355], while the lower peak could be explained by increased sales of commercial poultry due to competition with the availability of cheap guinea fowl eggs during this period of the year [265]. The highest peak of incidence also corresponds to a colder and drier period in Nigeria, and these climatic conditions may allow a prolonged persistence of the viruses in the environment compared to the rest of the year. It was unfortunately not possible to confirm the yearly patterns in our studies because we did not have a continuous sample collection throughout the years and most of the samples were collected between November and March.

The hypothesis that trade and bird movement play a major role in the spread of virulent NDV in West Africa would also imply that these new lineages may not be restricted to the investigated countries. Indeed, similar strains were also reported in Mali [353] and Mauritania [241]. NDV was also found in 4.9% (119/2427) of the samples collected in live bird markets in Benin and Togo between end 2008 and end 2010 [266] but no sequences are yet available. It is however interesting to note that the strains enzootic in West and Central Africa have not been reported anywhere else on the globe, except for two strains detected in wild birds in a quarantine station in the USA. Most NDV (sub-)lineages are not restricted to

one region (see the **Introduction** section for an overview of the geographic repartition of NDV strains), and some, such as sublineage 4b, are found on every continent, except Oceania, likely due to the movement of racing pigeons. The fact that West and Central African countries import more poultry products (live chickens, chicken meat and eggs; FAO STAT) than they export, may reduce the probability of exporting the virulent NDV strains.

In **Chapter 5**, we have investigated another reservoir of NDV: wild birds and pigeons. While the majority of strains found in wild birds are avirulent ones, cormorants in North America are a reservoir of virulent lineage 3c strains [199], pigeons carry virulent sublineage 4b strains worldwide [337] and wild birds were suspected to be at the origin of sublineage 5b outbreaks in Western and Northern Europe [202]. From our results, wild birds do not seem to be a reservoir of virulent NDV viruses or to play an important role their spread in West Africa, as only one passerine was found to be positive in Côte d'Ivoire. However, most of the samples from wild birds were collected in wild bird reserves and it would be interesting to investigate the role of bridge species in farms with low biosecurity measures where more interactions between wild birds and potentially infected poultry may occur.

Analyses of wild bird samples nevertheless revealed the presence of avirulent lineage 1 strains in Nigeria and constitute the first report of lineage 1 in Africa, besides vaccine-like strains in commercial farms (**Chapter 5**). Phylogenetic analyses revealed that the lineage 1 strains belonged to a cluster found in domestic and wild ducks in the USA, Europe and China [192; 235; 370], which may suggest that these strains were introduced by migratory birds. Interestingly, lineage 1 strains were found in Spur-winged Geese, the same species carrying the LPAI H5N2 strains, and also in the Hadejia-Nguru wetlands in North-East Nigeria. This could suggest that birds belonging to the *Plectropterus* and *Dendrocygna* genus play a similar role in the epidemiology of NDV and AIV by interacting with Eurasian migratory birds during their overwintering in sub-Saharan Africa.

The spread of HPAI H5N1 westward to Western Europe in 2006 made the set-up of an active surveillance program in Luxembourg necessary. Fortunately, no HPAI H5N1 virus was detected during the active surveillance (2006-2011; data not shown). We took the opportunity of the availability of the samples to screen them for NDV (**Chapter 6**), as the NDV status in wild birds in Luxembourg was unknown so far and the genetic information available from European strains was still scarce. Interestingly, lineage 1 strains belonging to the same cluster as the Nigerian ones were found in ducks in Luxembourg in 2007 and 2008. Their clustering with strains from Common Teals from Finland may also suggest that they were introduced by

wild birds, but in Luxembourg as well as in Nigeria, trade cannot be excluded as this cluster is also found in domestic ducks in the USA and China.

In both countries, several clusters of sublineage 4b strains were detected in pigeons (**Chapters 5 and 6**). In Nigeria, pigeons constitute an important part of the "poultry" sector because they are often breed for meat [372], while the few domestic pigeons in Luxembourg are mainly reared as a hobby. The three pigeon strains from Luxembourg did not share a recent common ancestor and may have been separately introduced because sublineage 4b circulates in the neighbouring countries [203] and is considered enzootic in some parts of Europe [196; 197]. But once more, trade cannot be excluded because no information on the origin of the pigeons, *i.e.* if they were domestic or town pigeons, was available. In Africa, NDV has rarely been investigated in wild birds and only few sublineage 4b sequences from South Africa, Kenya and Nigeria are available. It is therefore difficult to understand their origin, and we can only be sure that at least three and two separate introductions of lineage 4 strains happened in Nigeria and South Africa respectively.

Interestingly, no LaSota-like strains were found in Nigerian wild birds despite its massive use in the local poultry industry, but they were found in Luxembourg which has a non-vaccination policy towards NDV (**Chapters 5 and 6**). In the USA, no vaccine strains were found in wild birds either despite their use in the domestic poultry sector [192], but vaccine strains have been detected in wild birds in India, Argentina [194] and China [247], suggesting that spread may happen but not on a regular basis.

While HPAI H5N1 virus seemed to have disappeared from West Africa due to efficient control measures and/or self-extinction of the virus, virulent NDV seems to be circulating enzootically in the region. Several factors could explain the differences observed. First, influenza A viruses have a greater direct impact on human health than NDV viruses. HPAI H5N1 virus has caused more than 350 deaths whereas only one fatality has been associated with NDV to date [252]. There is therefore less pressure from the public health sector to control the spread of virulent NDV. Apprehension from the general population also contributed to reporting HPAI H5N1 outbreaks occurring in villages (O. Oni, pers. comm.).

Second, strong control measures were taken to stop the spread of HPAI H5N1 virus. Compensation measures likely encouraged farmers to declare the outbreaks to the authorities, and even if control measures may not have been perfect, they doubtlessly helped in

containing its spread. However, no such measures are in place for the control of Newcastle disease.

Third, Newcastle disease may not always be considered as a big problem for the commercial poultry industry. Vaccination of pullets, layers, breeders and broilers is common, at least in Nigeria, and proper vaccination has been shown to prevent the most serious consequences of NDV in most cases. However, vaccination does not prevent infection, virus replication and shedding, especially when the vaccine and challenge strains are distantly related [251]. Furthermore, other species like turkeys, ducks and guinea fowls are likely more resistant to the disease. The intermingling of immune chickens or other poultry species more resistant in live bird markets or backyard farms could contribute to the spread of virulent NDV without obvious clinical signs.

To help reducing the impact of virulent NDV on the poultry sector in West and Central Africa, vaccination of village poultry has been initiated in several countries, e.g. Togo, Senegal, Benin, Mali [339], Ghana [399], Mozambique [400] and Malawi [401]. Although it is an obvious solution, part of the cost probably needs to be supported by the authorities. Better biosecurity measures are likely the best way to slow the spread of NDV. As a repercussion of HPAI H5N1 virus outbreaks, biosecurity measures have already been improved [402] but awareness of farmers, veterinarians and traders on the transmission routes of NDV should be strengthened to stop its spread.

Conclusions and Perspectives

Although influenza A viruses have been extensively studied over the last century in Europe, Asia and North America, the recent epidemics/epizootics and pandemics have shown that we still have many lessons to learn about their pathogenicity, host range restriction and epidemiology. Despite several studies carried on, we will likely never be able to ascertain how HPAI H5N1 arrived in Africa. However, recent studies have highlighted that AIV is circulating all year round in the region. Reassortments between Eurasian and African influenza viruses also occur and the African gene pool should now be better characterized in order to understand the ecological drivers of AIV in Africa.

The recent emergence of the swine-origin pandemic H1N1 virus and its worldwide spread has also created new opportunities for the emergence of mixed viruses with unpredictable properties. In addition, the role of pigs in the epidemiology of influenza A virus is poorly known in Africa, despite its implication for public health.

This work has also shown that new viral lineages can still be discovered, even in viruses such as Newcastle disease virus that has been fairly well studied, and that the genetic classification will need to be constantly updated. However, experimental studies with the new strains identified should be performed to better understand their true virulence and the level of protection provided by the currently used vaccines.

Our current knowledge about the extent of the geographic spread of the new virulent NDV strains in Africa is still substantially restricted. With the exception of Nigeria which has been more extensively studied, only a limited number of strains have been sequenced in other countries from West and Central Africa and narrow areas in these countries have been investigated. Neighbouring countries may also be infected by some of these new virulent strains, and if it is not the case, it would be interesting to understand why.

Global trade and movements are a playground for the spread pathogens. Hence a global market implies the necessity of global awareness to be prepared in case of spreading and introduction of viruses in a new region. Molecular epidemiology is a powerful tool to follow up these events and trace the viruses back to their origin to be able to prevent some future incidents from happening.

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Appendix

Appendix I: Résumé en français

1. Situation du sujet

La viande de volaille et les œufs constituent l'une des sources de protéines bon marché partout dans le monde et sont d'importantes sources de revenus pour de nombreuses familles dans les pays en voie de développement. Ils constituent également une partie importante du secteur agricole dans les pays industrialisés où des milliers d'oiseaux peuvent être élevés par un seul agriculteur. La production de volaille est cependant menacée par deux maladies virales principales : la grippe aviaire hautement pathogène et la maladie de Newcastle, ayant des implications d'ordre économique et de santé publique à travers le monde.

Le virus de la maladie de Newcastle (NDV) a été reconnu comme tel en 1926 après l'apparition de foyers en Indonésie et en Angleterre. Il circule dans le monde entier, notamment en Asie et en Afrique où il est enzootique. La grippe aviaire a été identifiée vers la fin du 19ème siècle. Les oiseaux sauvages constituent le réservoir des virus de l'influenza aviaire (AIV) faiblement pathogènes pouvant être transmis aux espèces domestiques ou à certains mammifères. Chez l'Homme, l'introduction de nouveaux virus ou de nouveaux réassortants a conduit à cinq pandémies depuis le début du 20ème siècle, suivies par des périodes de circulation de grippes saisonnières causant la mort de 250 000 à 500 000 personnes chaque année malgré la disponibilité de vaccins.

Ces deux maladies ont un impact énorme sur la production mondiale de volaille, soit directement en provoquant une mortalité élevée dans les troupeaux infectés, mais aussi indirectement par les coûts considérables des mesures de contrôle, comprenant l'abattage sanitaire, les paiements compensatoires, les restrictions commerciales et la vaccination, ou en mettant en péril la durabilité de la production avicole dans les pays en développement.

2. Objectifs

L'émergence du virus de l'influenza aviaire hautement pathogène (IAHP) H5N1 et sa propagation au-delà des frontières du continent asiatique en 2005 a conduit à une épizootie sans précédent. Sa propagation a surpris les pays d'Afrique de l'Ouest qui n'avaient jamais connu de foyers d'IAHP jusque-là. Le premier objectif de cette étude était donc de surveiller

l'état sanitaire de la volaille, principalement dans les fermes à faible niveau de biosécurité et sur les marchés, qui ont été les parties les plus touchées du secteur (**Chapitre 1**).

L'apparition de l'IAHP H5N1 au Nigeria, sans une avancée progressive vers le sud depuis l'Europe ou le Moyen-Orient, était inattendue et les oiseaux migrateurs ont donc été soupçonnés d'avoir introduit le virus en Afrique de l'Ouest. Par conséquent, le deuxième objectif de ce travail était d'étudier la possibilité d'introduction des virus de l'influenza aviaire par des oiseaux migrateurs (**Chapitre 2**). Nous avons ciblé des oiseaux sauvages et domestiques dans deux régions, l'une étant le site principal d'hivernage au Nigeria pour les oiseaux migrateurs.

Dans les régions où les virus de l'IAHP ne sont pas enzootiques, le NDV est considéré comme la menace virale principale pour le secteur aviaire, y compris en Afrique occidentale et centrale. Bien que des virus mésogènes et vélogènes aient été isolés par le passé, il n'y avait aucune information sur la composition génétique des souches circulantes. Il était donc nécessaire de les caractériser. Dans une étude préliminaire (**Chapitre 3**), nous avons identifié des souches virulentes de NDV qui diffèrent de ce qui avait été rapporté dans d'autres pays. Cependant, seules des séquences partielles ont pu être obtenues et ce n'était pas suffisant pour mettre en place une classification fiable. Par conséquent, la surveillance a été maintenue et élargie à d'autres pays pour améliorer la caractérisation de ces souches et avoir une meilleure compréhension de leur répartition géographique et de leur diversité génétique (**Chapitre 4**).

De même que le virus de l'influenza aviaire, le NDV a une écologie complexe et le virus peut être infectieux pour plus de 250 espèces d'oiseaux, y compris des espèces sauvages. Les oiseaux sauvages sont considérés comme le réservoir des souches avirulentes de NDV qui peuvent parfois aussi être transmises à la volaille et évoluer vers des souches virulentes. De plus, certains oiseaux sauvages peuvent être porteurs de virus virulents et pourraient contribuer à leur propagation. Nous avons donc étudié le NDV chez les oiseaux sauvages en Afrique de l'Ouest, ce qui avait très peu été examiné auparavant (**Chapitre 5**).

La propagation du virus IAHP H5N1 en Europe a aussi stimulé la mise en place d'une surveillance active de la grippe aviaire au Luxembourg dans laquelle l'Institut d'Immunologie a été activement impliqué. La surveillance active et passive pour le NDV ont également été récemment encouragées par l'Union Européenne. Le dernier objectif de cette étude était donc de mettre en place la surveillance du NDV au Luxembourg en tirant parti des échantillons collectés lors de la surveillance AIV pour évaluer son statut (**Chapitre 6**).

3. Méthodologie générale

Pour chaque étude, des écouvillons trachéaux et/ou cloacaux ainsi que des fèces fraiches ou des organes *post-mortem* ont été prélevés. Les écouvillons et fèces ont été conservés dans du milieu de transport pour virus. Après extraction de l'ARN viral grâce à des kits commerciaux, l'ARN viral a été rétrotranscrit en ADN complémentaire et amplifié par réactions de polymérisation en chaine (PCR) avec des amorces spécifiques aux virus ciblés. La visualisation des résultats de PCR a été réalisée par électrophorèse sur gel d'agarose ou dans le cas de PCR en temps réel, par la mesure de la fluorescence émise lors de l'amplification des brins d'ADN. Ensuite, les gènes d'intérêt ont été amplifiés par PCR et séquencés par la méthode de Sanger et Coulson qui utilise l'incorporation aléatoire de didéoxyribonucléotides marqués avec traceurs fluorescents.

Les séquences obtenues ont été vérifiées en examinant visuellement les électrophérogrammes obtenus, assemblées par comparaison à une séquence de référence et alignées à l'aide de différents programmes informatiques. Les séquences ont ensuite été comparées à celles disponibles dans diverses banques de données, notamment la GenBank, au niveau nucléotidique et au niveau des acides aminés. Les relations phylogénétiques ont été calculées grâce à des analyses basées sur les distances génétiques entre paires de sequences (Neighbour-Joining) ou par des méthodes bayésiennes.

4. Résultats

4.1 La surveillance active de la grippe aviaire en Afrique de l'Ouest: aucune autre preuve de la circulation du H5N1 après 2008

L'arrivée de l'IAHP H5N1 en Afrique en 2006 a nécessité la mise en place d'un système de surveillance. Un total de 3163 échantillons nigérians et 1396 échantillons camerounais ont été testés par RT-PCR pour l'influenza aviaire entre 2006 et 2011. Parmi les échantillons collectés au Nigéria, 48.6% (143/294) en 2006 et 3.8% (30/786) en 2007 se sont avérés positifs. Seuls 103/173 des échantillons positifs pour la PCR détectant le gène M étaient positifs lors de la PCR spécifique pour le gène H5, mais aucun n'était positif pour le gène H7. Dans la plupart des cas (35/40 des emplacements testés), au moins un des échantillons était positif H5. Le séquençage, suivi de l'analyse phylogénétique des gènes HA, NA, M, NP et

NS de 47 échantillons, ont montré que tous les gènes de ces souches appartenaient au groupe A, un sous-groupe de la clade 2.2 constituée des souches H5N1 qui se sont répandues depuis l'Asie vers la Russie, l'Europe et le Moyen-Orient. Seules cinq souches en provenance d'une même ferme appartenaient au groupe dit $AC_{HA/NS}$ composé de virus réassortants. Les souches provenant d'une même ferme ou d'un même marché étaient toujours plus proches entre elles, sauf dans le cas d'un marché dans lequel une plus grande diversité génétique a été observée, soulignant le rôle joué par le commerce dans l'expansion de l'épizootie de l'IAHP H5N1.

4.2 Le virus réassortant de l'influenza aviaire faiblement pathogène de sous-type H5N2 dans les oiseaux sauvages africains

Afin de déterminer le rôle potentiel des oiseaux sauvages sur la présence et la persistance du virus de la grippe aviaire en Afrique, nous avons testé 805 oiseaux sauvages et 747 oiseaux domestiques dans deux régions au Nigeria, à savoir la réserve "Amurum Forest Reserve" située dans l'état de Plateau et la zone humide "Hadejia-Nguru wetlands" dans l'état de Yobe. Nous avons détecté le virus de l'influenza aviaire faiblement pathogène (IAFP) du sous-type H5N2 chez trois Oies armées de Gambie (*Plectropterus gambensis*) dans la zone humide d'Hadejia-Nguru. Les analyses phylogénétiques ont révélé que tous les segments des virus H5N2 faiblement pathogènes étaient plus étroitement apparentés à ceux récemment découverts chez les oiseaux sauvages et domestiques en Europe, à l'exception du segment NS. Le segment NS quant à lui, était plus étroitement apparenté aux segments NS d'AIV provenant de l'Afrique du Sud et la Zambie. La détermination du temps jusqu'à l'ancêtre commun le plus récent pour chaque segment du virus ainsi que les analyses phylogénétiques suggèrent que les virus H5N2 faiblement pathogènes nigérians trouvés chez les oiseaux sauvages sont issus d'un évènement de réassortiment impliquant des virus plus récemment introduits d'Eurasie, probablement par des oiseaux migrateurs intercontinentaux, et de virus circulant en Afrique depuis un certain temps. En effet, le gène NS semble circuler en Afrique depuis au moins 7 ans.

Fait intéressant, les gènes HA et NA des virus séquencés forment une branche sœur avec ceux de souches d'IAHP H5N2 trouvées dans les mêmes espèces d'oiseaux sauvages dans la même région un an plus tôt. Cependant, leurs gènes ne sont pas les plus apparentés, suggérant que leur présence dans la zone humide est le résultat de deux introductions distinctes. La présence d'influenza aviaire faiblement pathogène du sous-type H5N2 chez les oiseaux

sauvages dans les zones humides de Hadejia-Nguru où les oiseaux sauvages et les volailles domestiques se côtoient fréquemment offre de nombreuses possibilités pour la transmission à d'autres espèces avec le risque potentiel de générer des virus de l'IAHP après circulation chez les volailles.

4.3 Le virus de la maladie de Newcastle en Afrique occidentale: nouvelles souches virulentes identifiées dans les exploitations non commerciales

Quarante-quatre souches de NDV obtenues entre 2002 et 2007 à partir de différentes espèces de volaille au Nigeria, au Niger, au Burkina Faso et au Cameroun ont été analysées phylogénétiquement sur la base de la séquence partielle du gène de la protéine de fusion. Trois souches du Cameroun (2007) et du Nigeria (2005, 2006) faisaient partie de la lignée 1 et étaient très proches de la souche vaccinale Queensland V4. Dix-sept séquences provenant d'échantillons du Nigéria et du Burkina Faso et principalement de fermes commerciales étaient phylogénétiques très proches de la souche vaccinale LaSota couramment utilisée comme vaccin vivant, alors qu'une seule appartenait à la lignée 3. Deux souches virales détectées chez un pigeon et un perroquet appartenaient à la lignée 4b circulant principalement chez les pigeons dans le monde entier. Vingt-et-une souches provenant d'exploitations à faibles niveaux de biosécurité et de marchés ont été rassemblées en trois nouveaux groupes au sein de la lignée 5, provisoirement appelés 5f, 5g et 5h. Sur base de la séquence en acides aminés du site de clivage de la protéine F, ces souches 5f, 5g et 5h ont été prédites virulentes. Le groupe 5f a été détecté au Nigéria et au Niger, le groupe 5g au Nigéria, au Niger et au Burkina Faso alors que le groupe 5h a uniquement été trouvé au Burkina Faso. Les distances génétiques entre les sous-lignées 5 déjà établis et ceux nouvellement définis laissaient suggérer que ces dernières devraient peut-être être considérées comme une lignée à part entière, mais l'information génétique disponible n'était pas suffisante pour en être certain. Leur grande diversité génétique ainsi que leur présence dans trois pays d'Afrique subsaharienne suggèrent que ces nouvelles lignées représentent des variantes du NDV indigènes de l'Afrique de l'Ouest.

4.4 La diversité génétique du virus de la maladie de Newcastle chez les volailles en Afrique occidentale et centrale

Afin d'améliorer notre compréhension de la diversité génétique et de la répartition géographique des souches virulentes de NDV en Afrique occidentale et centrale, un total de 3610 échantillons provenant du Nigéria, du Cameroun, de la République Centrafricaine et de la Côte d'Ivoire ont été analysés pour la présence de NDV entre 2006 et 2011. 4.3% (157/3610) des échantillons ont été testés positifs et 96 séquences complètes et 5 séquences partielles du gène de la protéine F ont été obtenues et analysées. Toutes les séquences proches des souches vaccinales Queensland V4 ou B1 étaient originaires du Cameroun. Une analyse comparative de la diversité génétique des souches virulentes de NDV provenant de l'Afrique occidentale et centrale avec toutes les souches séquencées à ce jour a suggéré la définition et la répartition de ces souches en trois nouvelles lignées 7, 8 et 9, chacune subdivisées en 2 sous-lignées. Les analyses phylogénétiques ont révélé que plusieurs (sous-)lignées se retrouvent dans tous les pays, sauf en République Centrafricaine, où seul le groupe 8b a été identifié. Au Cameroun, neuf souches sont liées aux vaccins, mais une souche 8a a également été trouvée. Les lignées 8a, 9a et 9b sont présentes en Côte-d'Ivoire, et les lignées 7a, 7b, 8a et 9a ont été trouvées au Nigeria. Au Nigeria, le pays le plus largement échantillonné dans cette étude, plusieurs sous-lignées ou plusieurs groupes de souches au sein d'une même sous-lignée ont été détectés dans un seul et même état, suggérant une propagation au sein du pays ainsi qu'au-delà des frontières.

4.5 La diversité génétique des virus de la maladie de Newcastle chez les oiseaux sauvages et les pigeons en Afrique de l'Ouest

La répartition géographique assez étendue des souches virulentes de NDV en Afrique occidentale et centrale a soulevé la question du rôle potentiel des oiseaux sauvages dans leur propagation. Plusieurs souches de la sous-lignée 4b ont été identifiées chez des tourterelles et des pigeons au Nigeria et en Afrique du Sud. Des anticorps contre le NDV ont déjà été signalés chez des espèces sauvages en Afrique du Sud, au Burkina Faso et au Nigeria, mais aucune information génétique n'était disponible.

Dans cette étude, 1710 échantillons ont été testés. Dix échantillons se sont avérés positifs et le gène de la protéine F a été séquencé. Des séquences presque identiques provenaient de cinq

individus de la même espèce, l'Oie armée de Gambie, ayant été échantillonnés dans la zone humide de Hadejia-Nguru au nord-est du Nigéria. Ces souches appartenaient à la lignée 1, mais contrairement aux séquences obtenues précédemment en Afrique, elles étaient plus proches des souches sauvages circulant chez les canards domestiques et sauvages dans le monde entier. Quatre souches provenant de pigeons étaient les plus étroitement apparentées aux souches de la sous-lignée 4b déjà découvertes au Nigéria dans notre étude précédente, mais différentes de celles retrouvées dans d'autres états nigérians ou en Afrique du Sud. Ceci suggère que plusieurs introductions ont eu lieu en Afrique. Enfin, une souche appartenant à la nouvelle lignée 9b a été détectée chez un Tisserin gendarme (*Ploceus cucullatus*) retrouvé mort en Côte d'Ivoire. Il est probable que cet oiseau ait été infecté par contact direct ou indirect avec des volailles malades car des cas similaires ont été recensés chez des poulets dans la région environnante de Côte-d'Ivoire.

4.6 La caractérisation du virus de la maladie de Newcastle au Luxembourg de 2006 à 2010

En Europe, la maladie de Newcastle n'est pas considérée comme enzootique chez les oiseaux domestiques. La surveillance est néanmoins importante car les oiseaux sauvages constituent un réservoir naturel du virus. Des souches avirulentes et virulentes de NDV sont détectées chaque année en Europe.

Un total de 1131 échantillons provenant d'oiseaux sauvages et domestiques collectés entre 2006 et 2010 au Luxembourg ont été testés pour le NDV. Neuf échantillons se sont avérés positifs, correspondant à une prévalence de 0,8% au cours de la période 2006-2010. Trois souches avirulentes appartenant à la lignée 1 ont été détectées chez des canards sauvages en 2007 et 2008. La similarité de ces souches suggère que le virus pourrait avoir survécu et s'être disséminé localement. D'autre part, il est possible que ces souches aient été introduites séparément, notamment lors de mouvements migratoires d'oiseaux porteurs de ces souches. Deux souches avirulentes de la lignée 2 et très proches de la souche vaccinale LaSota ont été détectées chez un canard et un cormoran, insinuant une propagation de souches vaccinales en dépit de la politique de non-vaccination au Luxembourg. Parmi les oiseaux domestiques, un seul poulet était porteur d'une souche de la lignée 2 différente du vaccin LaSota et présentant un site de clivage de la protéine F GGQGR*L peu souvent rencontré mais indicatif d'une souche avirulente. Enfin, trois souches de la sous-lignée 4b ont été révélées chez des pigeons.

Le fait que ces trois souches ne sont pas directement apparentées les unes aux autres suggère qu'elles ont été introduites séparément au Luxembourg.

5. Discussion

Les résultats de la surveillance pour la grippe aviaire chez les oiseaux domestiques ont confirmé l'absence de l'IAHP H5N1 après 2008 au Nigéria. Le séquençage et les analyses phylogénétiques effectuées sur les souches H5N1 détectées en 2006 et 2007 ont confirmé la prédominance du groupe A en 2006, au moins dans le sud-ouest du Nigéria, mais ont aussi montré la co-circulation des groupes A et $AC_{HA/NS}$ en 2007 jusqu'à la fin de l'épizootie. Nous n'avons pas trouvé de souches du groupe C, rarement été détectée au Nigeria mais responsable de la majorité des cas répertoriés dans les autres pays d'Afrique, ni du groupe B qui a été détecté seulement dans le sud du Nigéria pendant une courte période alors qu'il circule sans interruption en Egypte depuis 2006. À ce jour, aucune explication n'a pu être donnée quant à l'apparente prospérité de certaines souches alors que d'autres semblent avoir disparu assez rapidement. D'autre part, notre surveillance n'a pas détecté la circulation d'AIV au Nigéria après octobre 2007, alors que des cas ont été signalés en juin et juillet 2008 dans le nord du pays. Ceci suggère que la prévalence était probablement faible et/ou que la surveillance en place n'était pas assez efficace pour détecter de faibles niveaux de circulation. Cependant, il semble que les virus IAHP H5N1 ont été éradiqués avec succès de la région puisqu'aucun échantillon n'était positif et qu'aucun autre rapport n'a mentionné leur présence depuis 2008.

Comme le suggère la diversité des souches d'IAHP H5N1 provenant dans un même marché, le mouvement d'animaux et le commerce ont probablement a eu un rôle majeur dans la dissémination de H5N1 au Nigeria. Il a également été suggéré que les marchés de volailles vivantes situés le long des frontières ont permis aux virus de se propager au-delà des frontières. Par contre, la manière dont a été introduit l'IAHP en Afrique alimente toujours les débats et le commerce et les oiseaux migrateurs ont été incriminés.

La détection de virus H5N2 faiblement pathogènes, porteurs de segments phylogénétiquement proches de ceux circulant en Europe ainsi que du segment NS présent dans d'autres virus d'influenza A en Afrique, suggère que des virus d'influenza A ont pu effectivement être importés en Afrique depuis l'Eurasie, mais surtout que l'AIV circule de manière continue en Afrique, contrairement à ce que était accepté jusqu'alors. Il semble que

l'affluence dans des sites particuliers (principalement en saison sèche) d'oiseaux migrateurs intercontinentaux et d'oiseaux africains partiellement migrateurs, ainsi qu'un période de nidification plus étalée chez les oiseaux africains soient des facteurs important pouvant compenser partiellement l'instabilité plus grande du virus dans un milieu moins favorable à sa survie.

La maladie de Newcastle pose une menace plus importante sur la production avicole en Afrique centrale et occidentale que l'AIV. Jusqu'à présent, très peu de données sur la composition génétique des souches circulantes étaient disponibles. Nous avons montré que de nouvelles souches virulentes de NDV étaient responsables des foyers rencontrés dans la zone étudiée. Leur distance génétique par rapport aux autres souches de NDV connues, leur diversité génétique et leur dispersion géographique suggèrent que ces souches ont probablement évolué localement, circulent depuis un certain temps dans la région et que le commerce et le mouvement d'animaux contribuent à leur propagation. Par contre, la nature des souches trouvées chez les oiseaux sauvages semble indiquer que ceux-ci ne contribuent que très peu à la dispersion des souches virulentes de NDV. Cependant, il serait intéressant d'étudier des espèces sauvages qui ont des rapports fréquents avec des oiseaux domestiques dans des fermes où des cas de la maladie de Newcastle ont été déclarés, pour corroborer cette hypothèse.

La détection de souches vaccinales de NDV chez des espèces sauvages aquatiques au Luxembourg, alors que la vaccination n'y est pas autorisée, pose la question du transfert du virus depuis la volaille vers le réservoir sauvage. Seulement quelques cas similaires ont été démontrés jusqu'à présent, bien que l'utilisation de vaccins vivants soit une pratique courante. Ceci suggère que ces cas sont probablement rares, mais l'impact sur la population d'oiseaux sauvages devrait être évalué afin de déterminer la nécessité de mesures prévenant la dispersion de ces souches vaccinales. De plus, l'introduction de virus NDV virulents au Luxembourg, qui n'a plus enregistré de cas depuis un certain temps, est possible à travers le mouvement local d'oiseaux tels que les pigeons, et il convient de continuer la surveillance afin d'assurer le statut de pays exempt de la maladie de Newcastle.

6. Conclusion et perspectives

Bien que le virus de l'influenza A ait été largement étudié au cours du siècle précédent en Europe, en Asie et en Amérique du Nord, les épidémies/épizooties et les pandémies récentes

nous ont montré que nous avons encore beaucoup de leçons à apprendre au sujet de sa pathogénicité, de la diversité de ses hôtes et de son épidémiologie. Malgré de nombreux efforts, nous ne serons probablement jamais en mesure de déterminer comment le virus de l'influenza hautement pathogène a été introduit en Afrique. Cependant, l'impressionnante motivation scientifique suscitée après son introduction a permis de mettre évidence que l'AIV circule de manière continue en Afrique. De plus, il apparaît désormais que des réassortiments entre des virus des pools africain et eurasien ont lieu. Le patrimoine génétique africain devrait maintenant être mieux caractérisé dans le but de comprendre les facteurs écologiques de l'influenza aviaire en Afrique.

L'apparition récente du virus de la grippe pandémique H1N1 et sa propagation mondiale a également ouvert de nouvelles opportunités pour l'émergence de virus mixtes avec des propriétés imprévisibles. Par ailleurs, le rôle des porcs dans l'épidémiologie de la grippe A est mal connu en Afrique, en dépit de ses implications pour la santé publique.

Ce travail a montré que de nouvelles souches virales peuvent encore être découvertes, même pour un virus tel que celui de la maladie de Newcastle qui a été assez bien étudié, et que la classification génétique devra être constamment mise à jour. Cependant, des études expérimentales avec les nouvelles souches identifiées devraient être réalisées afin de mieux comprendre leur virulence et le niveau de protection offert par les vaccins actuellement disponibles.

Nos connaissances actuelles sur l'ampleur de la répartition géographique des nouvelles souches virulentes de NDV en Afrique est encore considérablement limitée. Excepté pour le Nigeria, qui a été plus largement étudié, seul un nombre restreint de souches ont été séquencées dans d'autres pays d'Afrique occidentale et centrale. Les pays voisins sont également susceptibles d'avoir été infectés par certaines de ces nouvelles souches virulentes, et si ce n'est pas le cas, il serait intéressant de comprendre pourquoi.

Le commerce et les déplacements internationaux sont un terrain idéal pour la dissémination d'agents pathogènes. Cela implique la nécessité d'une sensibilisation mondiale afin d'être prêts en cas de propagation et d'introduction de virus dans une nouvelle région. L'épidémiologie moléculaire est un outil puissant pour assurer le suivi de ces événements et trouver leur origine pour finalement être en mesure de prévenir des incidents futurs.

Appendix II: List of publications, communications and training provided during the course of the PhD

1. Publications

● **Snoeck CJ**, M Marinelli, Charpentier E, Sausy A, Conzemius T, Losch S, Muller CP. Genetic diversity of Newcastle disease virus in Luxembourg: various risks for wild and domestic birds. Submitted to Applied and Environmental Microbiology.

● 2012. **Snoeck CJ**, Komoyo FS, Bonya MP, Okwen MP, Nakouné E, LeFaou A, Muller CP. Epidemiology of Chicken Anemia Virus in Central African Republic and Cameroon. Virol J, 9:189.

● 2012. **Snoeck CJ**, Gerloff NA, Tanasa RI, Abad Morejón de Girón FX, Muller CP. Highly Pathogenic Avian Influenza Virus. In *BSL3 and BSL4 Agents: Epidemiology, Microbiology, and Practical Guidelines*, pp175-200. Edited by Elschner M, Cutler S, Weidmann M and Butaye P. Darmstadt: Wiley-Blackwell.

● 2012. **Snoeck CJ**, Muller CP. Influenza Virus: Highly Pathogenic Avian Influenza, Practical guidelines. In *BSL3 and BSL4 Agents: Epidemiology, Microbiology, and Practical Guidelines*, pp328-333. Edited by Elschner M, Cutler S, Weidmann M and Butaye P. Darmstadt: Wiley-Blackwell.

● 2011. **Snoeck CJ**, Adeyanju AT, De Landtsheer S, Ottosson U, Manu S, Hagemeijer W, Mundkur T, Muller CP. Reassortant low-pathogenic avian influenza H5N2 viruses in African wild birds. J Gen Virol. 92:1172-83.

● 2009. **Snoeck CJ**, Ducatez MF, Owoade AA, Faleke OO, Alkali BR, Tahita MC, Tarnagda Z, Ouedraogo JB, Maikano I, Mbah PO, Kremer JR, Muller CP. Newcastle disease virus in West Africa: new virulent strains identified in non-commercial farms. Arch Virol. 154:47-54.

● 2009. Ducatez MF, Martin AM, Owoade AA, Olatoye IO, Alkali BR, Maikano I, **Snoeck CJ**, Sausy A, Cordioli P, Muller CP. Characterization of a new genotype and serotype of infectious bronchitis virus in Western Africa. J Gen Virol. 90:2679-85.

2. Oral communications

● 2012. **Snoeck CJ**: Virulent Newcastle disease virus in West and Central Africa. Life Science PhD Day, Luxembourg, 11th-12th September 2012.

● 2011. **Snoeck CJ**: Reassortant LPAI H5N2 viruses in wild birds in Nigeria in 2008. Séance de communications courtes d'automne 2011 de la Société des Sciences médicales du Grand-Duché de Luxembourg, Strassen, Luxembourg, 16th November 2011.

● 2011. **Snoeck CJ**: Epidemiology of Chicken Anemia Virus in Central African Republic and Cameroon. 15th Saar-Lor-Lux Meeting on Virus research, Remich, Luxembourg, 7th September 2011.

● 2010. **Snoeck CJ**: Reassortant LPAI H5N2 viruses in wild birds in Nigeria in 2008. Joint 16th Annual Meeting of the National Reference Laboratories for AIV and NDV of European Member States, Vienna, Austria, 26th-27th May 2010.

● 2009. **Snoeck CJ**: Spread an evolution of avian influenza H5N1 in Africa. 5th European Meeting on Viral Zoonoses, Saint-Raphaël, France, 26th-29th September 2009.

● 2009. **Snoeck CJ**: Reassortant low pathogenic avian influenza H5N2 in wild birds, Nigeria. 13th Saar-Lor-Lux Meeting on Virus research, Nancy, France, 9th September 2009.

● 2009. **Snoeck CJ**: Newcastle disease virus in West Africa: new virulent strains identified in non-commercial farms. 8th International Congress of Veterinary Virology, Budapest, Hungary, 23rd-26th August 2009.

● 2008. **Snoeck CJ**: Newcastle disease virus in West Africa: new virulent strains identified in non-commercial farms. 12th Saar-Lor-Lux Meeting on Virus research, Remich, Luxembourg, 12th September 2008.

3. Posters

● 2012. **Snoeck CJ**, Komoyo FS, Bonya MP, Okwen MP, Nakouné E, LeFaou A, Muller CP. Epidemiology of Chicken Anemia Virus in Central African Republic and Cameroon. 22nd Annual Meeting of the Society for Virology, Essen, Germany. 14th-17th March 2012.

● 2011. **Snoeck CJ**, Adeyanju AT, De Landtsheer S, Ottosson U, Manu S, Hagemeijer W, Mundkur T, Muller CP. Reassortant low-pathogenic avian influenza H5N2 viruses in African wild birds. Life Sciences PhD day, Luxembourg, Luxembourg, 13th September 2011.

● 2011. **Snoeck CJ**, Adeyanju AT, De Landtsheer S, Ottosson U, Manu S, Hagemeijer W, Mundkur T, Muller CP. Reassortant low-pathogenic avian influenza H5N2 viruses in African wild birds. Journée Scientifique des doctorants de l'Ecole Doctorale BioSE, Nancy, France, 26th January 2011.

● 2009. **Snoeck CJ**, Ducatez MF, Owoade AA, Maikano I, Tahita MC, Mbah PO, Martin AM, Cordioli P, Muller CP. Definition of New Avian Viruses Variants in West Africa During Active and Passive Surveillance. International Meeting on Emerging Diseases and Surveillance, Vienna, Austria

4. Training provided during the PhD period

● 2012 (9 weeks) : training of a PhD student (O.A., University of Ibadan, Nigeria)

● 2012 (5 weeks) : training of a technician (K. V., Institut Pasteur du Laos, Laos)

● 2011 (8 weeks) : training of a PhD student (O.O., University of Abeokuta, Nigeria)

● 2011 (6 weeks) : training of a PhD student (J.A., University of Zaria, Nigeria)

● 2011-2010 (10 months) : training and supervision of a Master student (M.M., Master in Cellular and Molecular Biology, Università di Tor Vergata, Rome, Italy)

● 2010 (6 weeks) : training of visiting scientist (B.P.M., Cameroon)

● 2009 (4 weeks) : training of visiting scientist (A.A.O., University of Ibadan, Nigeria)

● 2009 (10 weeks) : training of Master student (R.R. K., India)

● 2009 (3 weeks) : training of 5 Master students (H.A., A.S., B.O., J.G., I.O.N, University of Ibadan, Nigeria) at the University of Ibadan, Nigeria

● 2009 (6 weeks) : training of technician (G.F.K, Institut Pasteur de Bangui, Central African Republic)

● 2008 (4 weeks) : training of visiting scientist (E.C., Central Laboratory for Animal Diseases, Bingerville, Côte d'Ivoire)

● 2008 (12 weeks) : training of a Master student (A.T.A., University of Ibadan, Nigeria)

● 2008 (4 weeks): training of visiting scientist (A.A.O., University of Ibadan, Nigeria)

Appendix III: Additional publications

Journal of General Virology (2009), **90**, 2679–2685 **DOI** 10.1099/vir.0.012476-0

Characterization of a new genotype and serotype of infectious bronchitis virus in Western Africa

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Between 2002 and 2007, more than 1000 chickens from commercial farms, live bird markets and backyard farms in Nigeria and Niger were tested for the presence of the infectious bronchitis virus (IBV) genome. Phylogenetic analysis of full-length sequences of the spike 1 (*S1*) gene revealed a new genotype of IBV that we refer to as 'IBADAN'. The minimum genetic distance to the closest 'non-IBADAN' strains (UK/7/93 at the nucleotide level; H120 and M41 at the amino acid level) reached 24 and 32 % at the nucleotide and amino acid levels, respectively. The full genome of the IBADAN reference strain (NGA/A116E7/2006) had a genetic distance of 9.7–16.4 % at the nucleotide level with all available fully sequenced strains. As IBV S1 plays a major role in antigenicity, the antigenic relatedness of NGA/A116E7/2006 was compared with strains of other serotypes. NGA/A116E7/2006 did not cross-react with antisera against IT02, M41, D274, Connecticut or 793/B strains in virus neutralization assays. NGA/A116E7/2006 cross-reacted with the QX-like strain ITA/90254/2005 but only to a low level (antigenic relatedness of 33 %), suggesting that IBADAN also represents a new serotype. A comparison of *S1* sequences identified several amino acids that may play a role in IBV antigenicity. Despite the absence of obvious clinical signs in poultry infected by IBADAN strains, it is important to test the cross-protection of current vaccine strains.

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INTRODUCTION

The recent emergence of a coronavirus variant causing severe acute respiratory syndrome (SARS) in humans has renewed interest in the virus family *Coronaviridae*. Coronaviruses comprise three genetic groups, two of which (groups 1 and 2) contain viruses that are pathogenic in humans. Turkeys can be infected by group 2 as well as group 3 turkey coronaviruses (Lai & Holmes, 2001). Group 3 viruses such as infectious bronchitis virus (IBV) (Cavanagh, 2000; Enjuanes *et al.*, 2000; Lai & Holmes 2001), the first coronavirus to be discovered, occur only in birds. So far, group 3 viruses have not been found in humans, but phylogenetic analysis of SARS-coronavirus has shown that its genome contains sequences that seem to be of group 3 origin (Stavrinides & Guttman, 2004). IBV was first found in the USA in 1930 and has since been reported from most countries throughout the four continents of America (Johnson & Marquardt, 1975), Europe (Capua *et al.*, 1994; Cavanagh & Davis, 1993;

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The GenBank/EMBL/DDBJ accession numbers for the complete genome sequences of NGA/A116E7/2006 and ITA/90254/2005 are FN430415 and FN430414, respectively. Full *S1* and *N* gene sequences as well as partial *S2* gene sequences are under accession numbers FN182243–FN182283.

Details of the PCR conditions are available with the online version of this paper.

Gough *et al.*, 1992), Asia (Wang *et al.*, 1997) and Australia (Ignjatovic & McWaters, 1991; Lohr, 1976). Except for 793/B (or 4/91) (Cavanagh & Naqi, 2003), IBV genotypes are rarely reported to spread from one continent to another (Kusters *et al.*, 1987; Zanella *et al.*, 2003).

IBV is an enveloped, positive-strand RNA virus with a genome of about 27 kb. The nucleocapsid protein is the most conserved gene of IBV, whilst the spike 1 (S1) subunit of the spike protein gene is the most variable (Cavanagh & Naqi, 2003). This subunit is responsible for inducing neutralizing and serotype-specific antibodies. Mutations within this genome region may therefore result in the emergence of new variants against which vaccines are no longer protective (Moore *et al.*, 1998). IBV causes significant economic losses, mostly because of reduced productivity rather than bird mortality (Cavanagh & Naqi, 2003). The virus primarily infects the respiratory tract, kidneys and oviduct (Cavanagh & Naqi, 2003). Recent reports suggest a shift in tissue tropism (Liu & Kong, 2004; Liu *et al.*, 2006; Yu *et al.*, 2001; Zhou *et al.*, 2004) and an extended host range into new bird species reared close to domestic fowl. For instance, IBV was recently detected in Chinese peafowl (*Pavo*), guinea fowl (*Numida meleagris*), partridge (*Alectoris*) and teal (*Anas*) (Cavanagh, 2005). The differential diagnosis of the disease represents a challenge with respect to other respiratory diseases caused by *Mycoplasma gallisepticum* (chronic respiratory disease), infectious laryngotracheitis virus, *Haemophilus paragallinarum* (infectious coryza) and Newcastle disease virus.

In Africa, IBV has been reported only in Morocco in 1982–1983 (el Houadfi & Jones, 1985) and in Egypt in 2003 (Abdel-Moneim *et al.*, 2006). Antibodies against the virus were reported from South Africa (Thekisoe *et al.*, 2003), Zimbabwe (Kelly *et al.*, 1994), Botswana (Mushi *et al.*, 2006) and Nigeria (Ducatez *et al.*, 2004; Owoade *et al.*, 2006), where a seroprevalence of 84 % was detected in 1059 commercial chickens in the south-western part of the country. Despite the high prevalence of IBV in West Africa, little is known about the molecular and serological characteristics of these strains. Recently, two new economically important field types of IBV were isolated in domestic poultry in Europe and in China: Italy-02 (IT02) and QX viruses (Beato *et al.*, 2005; Bochkov *et al.*, 2007). Here, we analysed group 3 coronavirus IBV in West African poultry and showed not only that common IBV strains such as 793/B-, Massachusetts-, D274- and B1648-like strains circulate in the region but also new strains with unusual serological characteristics.

METHODS

Field samples. In the present study, more than 1000 cloacal swabs and 40 lung samples of poultry from Niger (Maradi, Niamey, Tillaberi and Zinder provinces) and Nigeria (Oyo, Ogun, Lagos, Kano, Kaduna and Sokoto states) were analysed, collected between 2002 and 2006. Cockerels, broilers, pullets, layers and breeders were obtained from commercial flocks with 125–20 000 birds (3-weeks- to 2-years-old) and from slaughterhouses, as well as from live bird markets in Nigeria and backyard farms in Niger.

Virus cross-neutralization. Monospecific antisera against the viruses NGA/A116E7/2006, ITA/90254/2005 QX-like, M41, D274, Connecticut (Conn) and 793/B were produced following a standard immunization protocol (Gelb & Jackwood, 1998). Briefly, specific-pathogen-free (SPF) chickens were inoculated intratracheally with approximately 10^5 50 % egg infectious doses (EID_{50}) per bird. At 3 weeks post-inoculation, they received an intravenous injection of the same dose. After another 4 weeks, blood samples were collected and serum was harvested, pooled and inactivated at 56 °C for 30 min before being used in virus neutralization (VN) assays. Serum specific for IT02 was kindly provided by Dr Capua (IZS Padova, Italy).

To determine the antigenic relationships between the Nigerian NGA/A116E7/2006 strain (after seven passages in embryonated chicken eggs) and other reference strains, reciprocal β VN assays, with a fixed concentration of virus and serial dilutions of serum, were carried out. The field strains used included NGA/A116E7/2006 and ITA/90254/2005 (97.9 % nucleotide identities to QX IBV for the *S1* hypervariable region). The reference strains were IT02, D274, 793/B, M41 and Conn. VN tests were performed as described by Thayer & Beard (1998). Twofold serial dilutions of each antiserum were mixed with an equal volume of virus dilution containing 100 EID_{50} in 0.1 ml and incubated for 1 h at room temperature. Each serum–virus mixture was then inoculated in SPF chicken embryonated eggs by the allantoic sac route. Viruses were back-titrated in each VN test to confirm that 100 EID_{50} virus per 0.1 ml had been used. Chicken embryos were evaluated 24 h after inoculation for non-specific mortality and 1 week after inoculation to evaluate the presence of specific lesions, as an indication that the virus had not been neutralized by sera. End points corresponded to the serum dilutions that neutralized 50 % of the virus. End-point titres were calculated by the method of Reed & Muench (1938).

The VN end-point titres were used to calculate the percentages of antigenic relatedness, *r*, by the method of Archetti & Horsfall (1950). The *r* value is equivalent to the square root of $r_1 \times r_2$, where r_1 is the ratio of the heterologous titre with virus 2 to the homologous titre of virus 1, and r_2 is the ratio of the heterologous titre with virus 1 to the homologous titre of virus 2. The data were expressed as percentage *r* values. The *r* values determined by Archetti & Horsfall (1950) ranged from 0 for isolates that were antigenically unrelated to 100 % for isolates that were identical. Isolates with *r* values between 50 and 100 % were considered to be antigenically related (Gelb *et al.*, 1997).

RNA isolation and RT-PCR. RNA was extracted using a QIAamp viral RNA mini kit (Qiagen). RNA was eluted in 60 µl elution buffer. The extracted RNA was first reverse-transcribed with random primers and SuperScript III (Invitrogen). The cDNA was screened for the IBV genome using a highly sensitive nested-PCR specific for a constant region of the nucleocapsid protein gene (Akin *et al.*, 2001). In a first approach, a region of the *S1* gene (approx. 400 nt) was amplified from IBV-positive samples in a nested or semi-nested format (Adzhar *et al.*, 1997). The full *S1* gene was then amplified using previously published primers (Dolz *et al.*, 2006). Additional PCR primers were designed to amplify *S1* genes when previously published primers failed. Primers were also designed to amplify the full genome of the NGA/A116E7/2006 and ITA/90252/2005 QX-like strains. The PCR conditions are summarized in Supplementary Table S1 (available in JGV Online). All PCRs were performed in 25 µl final volume with 1 U Platinum *Taq* DNA polymerase (Invitrogen) per reaction. The equivalent of 0.5 µl of the reaction mix of the reverse transcription reaction or of the first PCR was transferred to a new tube for the first round or the nested reactions, respectively. All programmed cycling was performed in a thermocycler (Mastercycler Gradient; Eppendorf). PCR amplicons were analysed in a 1.5 % agarose gel (Ultrapure; Invitrogen), using

1× TAE electrophoresis running buffer and stained with ethidium bromide (15 µg in 100 ml agarose gel). IBV vaccine strain H120, kindly provided by Drs Palya and Penzes (CEVA Phylaxia Veterinary Biologicals, Budapest, Hungary), was used as a positive PCR control and to optimize the different PCRs.

Sequencing. PCR products were purified using a Jetquick PCR purification kit (Genomed) following the instructions of the manufacturer. DNA (10–100 ng) was sequenced in both directions with a Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) on a capillary sequencer (model 3100-Avant; Applied Biosystems) using the PCR primers (Eurogentech) as sequencing primers. Strains were designated as three-letter country code/sample ID/year.

Data analysis. Sequences were analysed using Seqscape version 2.5 (Applied Biosystems) and BioEdit programs (Hall, 1999). Sequences were aligned with CLUSTAL W (Chenna *et al.*, 2003). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0.2 (Kumar *et al.*, 2001). Phylogenetic analysis of nucleic acid and deduced amino acid sequences was done with the neighbour-joining method using the Kimura two-parameter model and pairwise deletion. Amino acid sequences were also analysed with the neighbour-joining method, with Poisson correction. Bootstrap values (1000 replications) were indicated on the trees. IBV sequences from all relevant reference strains available on GenBank were used for comparison.

RESULTS

Virus prevalence and disease symptoms

In Nigeria, 18 % (170/957) of the chickens tested were positive for IBV RNA: 7/40 (18 %) in slaughterhouses in 2002; 161/617 (26 %) in commercial farms in 2006; and 2/300 (1 %) in live birds markets in 2007. The virus was detected in 3–55-week-old commercial broilers and layers, in both chicken and turkey farms. In Niger, only 2 % (1/62) of backyard birds collected in 2006–2007 were IBV positive. In IBV-positive commercial farms, slight losses in productivity were observed, but no obvious clinical symptoms were identified.

When IBV NGA/A116E7/2006 was inoculated intranasally into 6-week-old SPF chickens, the animals developed no clinical symptoms but produced antibodies against the virus. These antibodies were used for cross-neutralization assays, described below.

Phylogenetic analysis of partial *S1* sequences

The hypervariable region of the *S1* gene (380 nt) was sequenced from all strains and compared with all relevant IBV sequences available in GenBank (Fig. 1). The 2002 strains clustered with either 793/B-type strains such as UK/7/93 (Z83979) or Massachusetts-type strains such as M41 (DQ664534; Fig. 1). Some Nigerian 2006 strains clustered with 793/B-like strains (NGA/254/2006, NGA/371/2006 and NGA/386/2006; Fig. 1), and others with the Belgian nephropathological strain B1648 (NGA/271/2006, NGA/279/2006 and NGA/324/2006), the D274-like strains

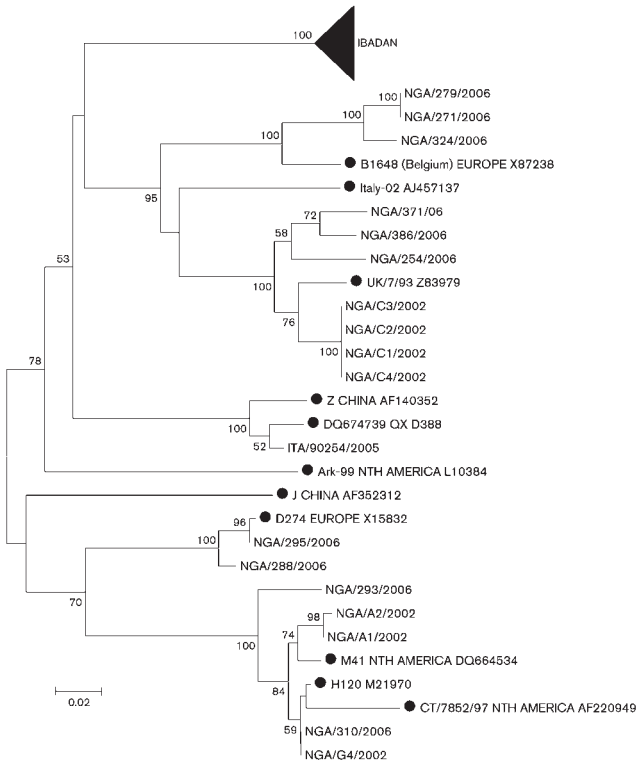

Fig. 1. Phylogenetic analysis of the hypervariable region of the IBV *S1* gene from strains from Nigeria and Niger at the nucleotide level. The tree was constructed with the neighbour-joining method (Kimura two-parameter) with 1000 bootstrap replicates. Only bootstrap values higher than 49 are shown. Filled circles indicate IBV reference strains. Bar, 0.02 nucleotide substitutions per site.

(NGA/288/2006 and NGA/295/2006) or with Massachusetts-like strains (NGA/293/2006 and NGA/310/2006; Fig. 1). Interestingly, one farm (SA, sample numbers 288, 293 and 295) was infected with two distinct strains related either to Massachusetts (NGA/293/2006) or to D274 (NGA/288/2006 and NGA/295/2006). The remaining 2006 Nigerian IBV strains (from the north and the south-west) formed a distinct cluster, which was designated 'IBADAN' (the name of the Nigerian city where the strain was first detected) (Fig. 1).

Phylogenetic analysis of complete *S1* sequences

The full *S1* gene sequence was available only from 30 strains from Nigeria and one strain from Niger. The IBADAN-like strains were compared with the ten reference sequences and all complete *S1* sequences available in GenBank, as well as with a recent QX strain (ITA/90254/2005), sequenced for the purpose of this study (Fig. 2). The three complete *S1* sequences obtained from farm SA (NGA/288/2006, NGA/293/2006 and NGA/295/2006) clustered together with a mean genetic distance between the three strains of 8.3 %, and with the D274 strain (Fig. 2).

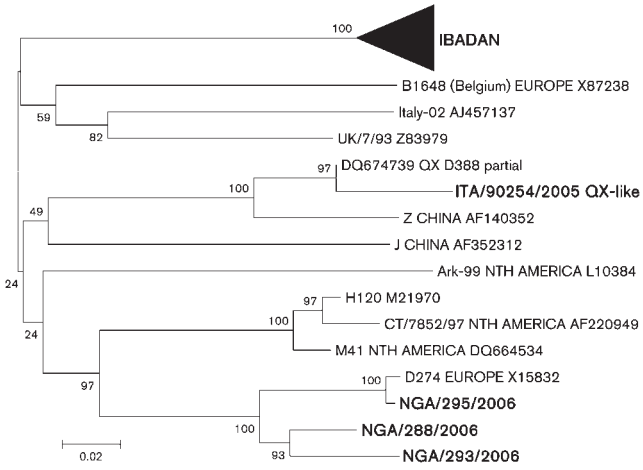

Fig. 2. Phylogenetic analysis of the full-length IBV *S1* gene from Nigeria and Niger strains at the nucleotide level. The tree was constructed with Poisson correction with 1000 bootstrap replicates. Only bootstrap values higher than 49 are shown. Ten IBV reference strains as well as ITA/90254/2005 were included. Strains sequenced in the present study are indicated in bold. Bar, 0.02 nucleotide substitutions per site.

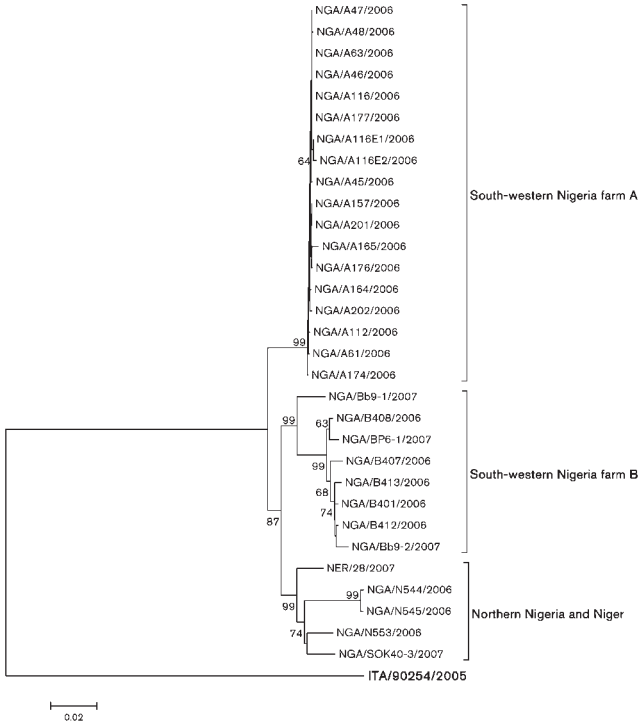

Fig. 3. Phylogenetic analysis of the full-length IBV *S1* genes from Nigerian and Niger IBADAN strains at the nucleotide level. The tree was constructed with the neighbour-joining method (Kimura two-parameter) with 1000 bootstrap replicates. Only bootstrap values higher than 49 are shown. ITA/90254/2005, shown in bold, was used to root the tree. Bar, 0.02 nucleotide substitutions per site.

The phylogenetic tree of the full-length *S1* sequences also confirmed the IBADAN cluster, supported by a bootstrap value of 100 at the main node (Fig. 2). These strains also formed a distinct cluster at the amino acid level (data not shown). Within the IBADAN cluster, the mean genetic distance reached 3.4 % (range 0–8.8 %) at the nucleotide level and 5.2 % (range 0–14.1 %) at the amino acid level. Seven amino acid positions were shared by all IBADAN strains and were not found in any other reference IBV sequence: I414, I463, S475, R491, E493, G512 and Y527 (comparison with the ten reference strains included in the phylogenetic analyses). The minimum genetic distance to the closest 'non-IBADAN' strain was 24 % [between NER/28/2007 and UK/7/93 (Z83979)] or 25 % [between NGA/A176/2006 and UK/7/93 (Z83979) or between NGA/SOK40-3/2007 and D274 (X15832)] at the nucleotide and amino acid levels, respectively.

As recombinations are relatively frequent in IBV, IBADAN sequence fragments of 100–600 nt were further compared phylogenetically with all available strains. In all cases, IBADAN strains branched separately, excluding any obvious recombination events in *S1*.

The new IBADAN strains formed location clusters at the nucleotide level (south-western Nigeria; northern Nigeria and Niger; Fig. 3) supported by high bootstrap values. Two farms, A and B, in the south-west of Nigeria were separated by less than 100 km and hosted viruses with 4.6–5.6 % Kimura distances. The genetic distance within the northern Nigerian farm was even higher (6.0 %), whilst it was much lower within farm B strains over a 9 month period (0.6–3.4 %).

After five passages in embryonated eggs, NGA/A116/2006 acquired a single-nucleotide, non-silent mutation (T689C, resulting in the amino acid change V230A). Two more mutations (one silent nucleotide change at A279C; one non-silent nucleotide change at A191G resulting in amino acid change E64G) were acquired after two additional passages.

The new Nigerian strains did not increase the worldwide maximal genetic diversity for the complete IBV *S1* genes at either the nucleotide or amino acid level.

Phylogenetic analysis of the full genome

The full-genome sequences of both the IBADAN strain NGA/A116E7/2006 and the QX-like strain ITA/90254/2005 were obtained. The phylogenetic analysis of all relevant full-length sequences is presented in Fig. 4. The genetic distance over the full-genome sequences ranged between 4.7 % [strain ArkDPI11 (EU418976) to strain 'serotype_California_99' (AY514485)] and 16.4 % [strain A2 (EU526388) to strain NGA/A116E7/2006 or 'serotype_California_99']. The genetic distances between the IBADAN strain NGA/A116E7/2006 and the ITA/90254/2005 strain reached 10 % at the nucleotide level. The

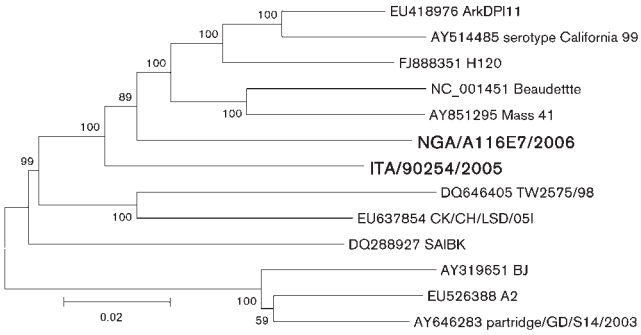

Fig. 4. Phylogenetic analysis of the full-length IBV genome from all 11 relevant strains available in GenBank as well as NGA/A116E7/2006 and ITA/90254/2005 (both shown in bold) at the nucleotide level. The tree was constructed with the neighbour-joining method (Kimura two-parameter) with 1000 bootstrap replicates. Only bootstrap values higher than 49 are shown. Bar, 0.02 nucleotide substitutions per site.

genetic distance of IBADAN and QX to other genotypes ranged from 9.7 and 10 % (ArkDPI11) to 16.4 and 14.1 % (A2), respectively.

Antigenicity of West African IBV

The antigenic properties of NGA/A116E7/2006 were compared by a virus cross-neutralization assay to ITA/90254/2005 and a number of reference strains (Table 1). Virtually no cross-neutralization of NGA/A116E7/2006 was observed with IT02, D274, 793/B, M41 or Conn antiserum (titres $\leqslant$1 : 22; Table 1). The VN titre of ITA/90254/2005 QX-like antiserum against NGA/A116E7/2006 virus was 1 : 256. NGA/A116E7/2006 antiserum did not neutralize IT02, D274, 793/B, M41 or Conn (titres $\leqslant$1 : 32), but showed some cross-reactivity with ITA/90254/2005 (VN titre 1 : 128). Titres against homologous strains were 1 : 300 for NGA/A116E7/2006 and 1 : 1024 for ITA/90254/2005 (Table 1). The calculated antigenic relatedness value, *r*, confirmed the absence of a relationship between NGA/A116E7/2006 and IT02, D274, 793/B, M41 or Conn ($r<10$). The value of *r* when comparing NGA/A116E7/2006 and ITA/90254/2005 was somewhat higher ($r=33$ %), but did not reach the 50 % threshold for antigenic relatedness between strains (Table 2).

NGA/A116E7/2006 and ITA/90254/2005, which cross-reacted antigenically, shared only 71 % nucleotide identity or 72 % amino acid identity over the complete *S1* gene.

DISCUSSION

In the south-west of Nigeria, up to 26 % of the poultry from commercial farms were infected with IBV. In northern Nigerian live bird markets and in Niger backyard poultry, IBV infections seemed to be less common. In a more systematic prevalence study (Ducatez *et al.*, 2004; Owoade *et al.*, 2006) conducted in Nigeria from 1999 to 2004, 84 % of the commercial poultry were positive for IBV antibodies. Most of the IBV strains characterized here grouped together (mean and maximal genetic distances of 3.4 and 8.8 %, respectively) and were distinct from all strains reported so far. No clear minimal genetic distance has been proposed to distinguish between IBV genotypes, unlike some other viruses such as measles virus (WHO, 2003) and hepatitis B virus (Norder *et al.*, 1992).

Recently, a new IBV genotype QX was proposed. For the 'original reference' strain D388 isolated in The Netherlands, only a short sequence was available (the *S1* hypervariable region). Therefore, we sequenced ITA/90254/2005 to generate the only full sequence of a QX-like strain currently available. This virus *S1* gene was separated by 14.3 % at the nucleotide level and 16 % at the amino acid level from its closest relative of a different genotype (Z China, AF140352). IBADAN *S1* was separated by 24 and 25 % at the nucleotide and amino acid levels, respectively, from UK/7/93 (Z83979), the closest strain of a different cluster.

S1 is the main antigenic protein of IBV, inducing neutralizing and serotype-specific antibodies (Cavanagh & Naqi, 2003). The 25 % genetic distance on an amino acid

Table 1. VN assay titres

Titres were obtained in reciprocal β VN assays (diluted serum, constant virus; Thayer & Beard, 1998).

Virus	Serum						
	NGA/A116E7/2006	ITA/90254/2005 QX-like	IT-02	M41	D274	Conn	793/B
NGA/A116E7/2006	1/300	1/256	1/22	$\leqslant$1/8	1/20	$\leqslant$1/8	$\leqslant$1/8
ITA/90254/2005 QX-like	1/128	1/1024					
IT-02	1/32		$>$1/512				
M41	$\leqslant$1/8			$>$1/512			
D274	1/16				$>$1/512		
Conn	$\leqslant$1/8					$>$1/512	
793/B	$\leqslant$1/8						$>$1/512

Table 2. Antigenic relatedness, *r* (%), of NGA/A116E7/2006

Virus 2	Virus 1: NGA/A116E7/2006
NGA/A116E7/2006	100
ITA/90254/2005 QX-like	33
IT-02	<10
M41	<10
D274	<10
Conn	<10
793/B	<10

level prompted us also to investigate the antigenic cross-reactivity of IBADAN strains with strains of other relevant serotypes. The IBADAN-like strain NGA/A116E7/2006 showed no antigenic cross-reactivity with any of the tested IBV serotypes and only a weak cross-reactivity with ITA/90254/2005 QX-like strain (Table 1). The relatively low antibody titre induced by the IBADAN-like strain partially explains some of the low level of antigenic relatedness (*r*) calculated for this new virus. Nevertheless, the VN data suggested that QX-like viruses may antigenically be the closest relatives of IBADAN. The comparison of the full genomes of NGA/A116E7/2006 and ITA/90254/2005 showed a diversity of 10 % at the nucleotide level. IBADAN was separate from any known full-genome IBV nucleotide sequence with a genetic distance ranging from 9.7 to 16.4 %. This very high global molecular diversity therefore further justifies the designation of a new genotype, IBADAN.

A comparison of the amino acid sequence of IBV S1 showed that V70, K95, V211 and S335 were shared by NGA/A116E7/2006 and ITA/90254/2005 but not by any of the other strains included in the VN assay (IT02, M41, D274, Conn and 793/B, which had A/T/I70, T/S/V95, A211 and N/K335). This observation suggests that any of these positions may explain the low level of cross-reactivity between NGA/A116E7/2006 and ITA/90254/2005. However, these amino acid positions are not unique for ITA/90254/2005 and NGA/A116E7/2006 strains – some of them are also found in B1648 and Z and J China (data not shown). These comparisons may provide some guidance for further antigenicity studies.

In Nigeria, breeder farms vaccinate 10-day-old chicks with a single dose of live-attenuated IBV vaccine (e.g. Massachusetts-like strains H120 and H52). Therefore, it cannot be fully excluded that some of the strains detected (e.g. NGA/A1/2002, NGA/A2/2002 and NGA/G4/2002 strains) were vaccine-derived. In one farm, strains were found that clustered with both putative vaccine strains (Massachusetts-like, NGA/293/2006; Fig. 1) and the wild-type strain D274 (NGA/288/2006 and NGA/295/2006), which could be an indication of vaccine failure. However, full *S1* sequences of NGA/293/2006 clustered with NGA/288/2006 and NGA/295/2006, which could be indicative of recombination between Massachusetts-like and D274-like strains.

In conclusion, we have presented here a set of new IBV strains from two countries in West Africa, which are genetically and antigenically clearly distinct from all other known IBV strains. We propose to call the new genotype and serotype IBADAN, according to the location where the reference virus was found. It is important to compare the pathogenicity of IBADAN strains with other circulating IBV strains and to test their sensitivity with respect to current vaccines.

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Epidemiology of chicken anemia virus in Central African Republic and Cameroon

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Abstract

Background: Although chicken anemia virus (CAV) has been detected on all continents, little is known about this virus in sub-Saharan Africa. This study aimed to detect and characterize CAV for the first time in Central African Republic and in Cameroon.

Results: An overall flock seroprevalence of 36.7% was found in Central African Republic during the 2008–2010 period. Virus prevalences were 34.2% (2008), 14.3% (2009) and 10.4% (2010) in Central African Republic and 39% (2007) and 34.9% (2009) in Cameroon. CAV DNA was found in cloacal swabs of 76.9% of seropositive chickens, suggesting that these animals excreted the virus despite antibodies. On the basis of VP1 sequences, most of the strains in Central African Republic and Cameroon belonged to 9 distinct phylogenetic clusters at the nucleotide level and were not intermixed with strains from other continent. Several cases of mixed infections in flocks and individual chickens were identified.

Conclusions: Our results suggest multiple introductions of CAV in each country that later spread and diverged locally. Mixed genotype infections together with the observation of CAV DNA in cloacal samples despite antibodies suggest a suboptimal protection by antibodies or virus persistence.

Keywords: Chicken anemia virus, Central African Republic, Cameroon, Antibodies, PCR, Phylogeny, Mixed infection

Background

Chicken anemia virus (CAV), the only member of the *Gyrovirus* genus in the *Circoviridae* family [1], was first discovered in the late 70's [2], but a retrospective study revealed that the virus circulated in chickens long before that [3]. Chickens are considered the only natural host of chicken anemia virus, although anti-CAV antibodies have also been detected in Japanese quails [4] but not in other domestic or wild bird species [4,5]. CAV is ubiquitous [6] and the virus seems to be particularly well adapted to its host [7].

The fecal-oral route constitutes probably the main mode of horizontal transmission [8], but CAV was also detected in feather shafts indicating that other modes of dissemination may be possible [9]. Experimental infections via the respiratory tract were also successful [10] but the relevance of such experiments in the field is still unclear. The virus can also be transmitted vertically from infected parents, either the male or female [11,12], irrespective of their antibody status [11,13,14], to their progeny. Seroconversion of specific pathogen-free chickens around the onset of lay without detectable virus in the flock suggested that CAV could be maintained in reproductive organs as a latent or persistent infection with low levels of replication, and become reactivated when the animal reaches sexual maturity [7,11,15].

The virus causes severe anemia, pale bone marrow, thymus atrophy and severe immunosuppression in 2–3 weeks old chickens if they are not protected by maternal antibodies [7]. In older animals infections usually result in subclinical disease that can also lead to economic losses in poultry farms due to reduced weight gain and increased susceptibility to secondary infections [16-19]. Co-infections with Marek's disease virus or infectious bursal disease virus (IBDV) may lead to a more complex and severe disease [20,21].

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Although the poultry sector is an important part of the economy in many African countries, CAV has only been reported without further details from South Africa [22] and more detailed studies were done by us and others in Nigeria [23-27]. In this study, we describe for the first time the presence and the genetic diversity of CAV in Central African Republic (CAF) and in Cameroon (CMR).

Results

Seroprevalence

In Bangui (CAF), anti-CAV antibodies were found in 147 chicken sera out of the 400 analyzed. In farms, 36.7% (29/79) of the flocks had at least one chicken with anti-CAV antibodies and there was little change throughout the 3 years of sampling: 34.8% (8/23) in 2008, 39.3% (11/28) in 2009 and 35.7% (10/28) in 2010 (Table 1). Four flocks had a seroprevalence below 25%, 5 flocks had a seroprevalence between 25 and 50%, and 20 flocks had a seroprevalence above 50% (Table 1). Most seropositive flocks for which the age was available became seropositive after week 5 (Figure 1A). Several farms were visited 2 or 3 times but no trend could be seen when comparing percentage of seropositive animals between collection time points. In the 2 live bird markets where sera were collected, 69% (68/98) of chickens were seropositive and the seroprevalence in each market was 67.9% (38/56) and 71.4% (30/42).

Virus prevalence

In CAF, CAV nucleic acids were detected in 45.8% (11/24) of the flocks in 2008, in 31.7% (19/60) in 2009 and in 15% (9/60) in 2010 (Table 2). Fourteen flocks had a prevalence below 25%, 7 flocks had a prevalence between 25 and 50%, and 18 flocks had a prevalence above 50% (Table 2). Similarly to the seroprevalence data, no trend in the percentage of virus infected animals was observed over the time in farms visited several times. CAV was also detected in 21.2% (42/198) of samples collected in live bird markets and 3 of the 5 markets were infected (prevalence of 27.8% (30/108), 15.5% (11/71) and 9.1% (1/11) over 21 months). For 400 animals, sera and swabs were available and 76.9% (113/147) of the antibody positive animals were PCR positive while all antibody negative animals (253/400) were PCR negatives.

Overall prevalences of CAV infections of 34.2% (26/76), 14.3% (59/412) and 10.4% (33/316) were found in 2008, 2009 and 2010 respectively in Central African Republic, compared to 39% (112/287) in 2007 and 34.9% (203/582) and 2009 in Cameroon.

In both countries, a total of 433 samples were positive for CAV nucleic acids and were submitted for sequencing.

Table 1 Seroprevalence in flocks from Central African Republic

Year	Seroprevalence (%)					Total
	0	> 0 and ≤ 25	> 25 and ≤ 50	> 50 and ≤ 75	> 75 and ≤ 100	
2008	15	1	1	2	4	23
2009	17	1	1	1	8	28
2010	18	2	3	3	2	28
Total	50	4	5	6	14	79

Seroprevalence is expressed as the number of flocks with a seroprevalence included in a percentage range.

Phylogenetic analyses of nucleotide sequences

Sequencing of the VP1 coding region was attempted for all CAV positive samples but only 228 sequences of at least 1281 nucleotides from the 433 positive samples were obtained. The phylogeny of these 228 VP1 gene sequences (1281 bp) revealed several clusters within group II or III in both countries (Figure 2). The 53 VP1 sequences from 20 chicken flocks and 3 live bird markets in Bangui formed 4 groups (CAF1 to CAF4; Figures 2 and 3). CAF1 strains were very similar to each other (maximal Kimura distance mKd 0.5%) and 1 strain CAF09-144 clustered outside CAF1 group (mKd 0.9% to CAF1). The CAF1 strains were most closely related to strains from Argentina, 2007–2008 (Figure 3). The CAF2 group consisted of only 2 strains (mKd 2.1%) that formed an isolated cluster within group II (Figure 3). Twenty-five strains formed the CAF3 group (mKd 0.8%; Figure 3). The CAF4 group contained 8 strains and was as diverse as CAF2 (mKd 2.1%), but the clustering of CAF09-153 was uncertain as shown by the discrepancies in the trees of Figures 2 and 3. All groups contained strains from farms and live bird markets, except CAF2 which contained strains from markets only. All strains from 2008 clustered in CAF1 and CAF3; 2009 strains were found in all groups and 2010 strains in CAF1 to 3.

One hundred seventy-five sequences from chickens in Cameroon clustered in 5 main groups (CMR1 to CMR5; Figures 2 and 4). CMR1 cluster included 104 strains from both 2007 and 2009 (mKd 1.4%), and a Nigerian strain CAV/Ejioku.NIE/11.02/107 (Figure 4A). CMR2 contained 11 strains from 2009 (mKd 0.2%; Figure 4B) and was most closely related to a Japanese strain and to the CAF3 cluster (Figure 2). The CMR3 group included 35 strains from 2009 (mKd 0.9%) and was most closely related to a Chinese strain CH_CK/05-01/HN/592 (Figure 4B). In addition, 1 strain CMR09-565.1 clustered outside CMR3 group (mKd to CMR3 of 1.3%; Figure 2). CMR4 cluster contained 21 very similar strains from 2007 only (mKd 0.2%). Two strains from 2009 clustered in CMR5 (mKd 0.1%; Figure 4B), and one strain (CMR09-314) clustered with Argentinean strains but did not intermingle with CAF1 strains (Figures 2 and 4B).

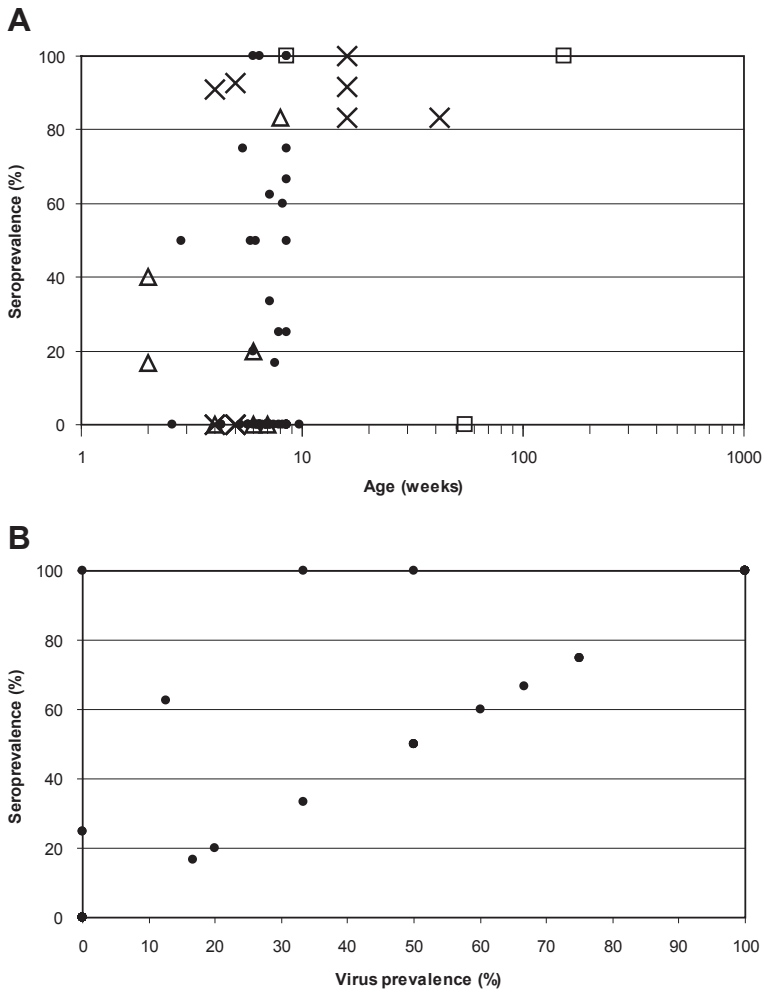

Figure 1 Anti-CAV antibody seroprevalence in CAF. (**A**) Age-dependent seroprevalence in broiler flocks (●) and layer flocks (□) in CAF and in broiler (Δ) or pullet and layer (x) flocks in Nigeria (from Owoade *et al.* [27]). (**B**) Relation between seroprevalence and virus DNA prevalence in chicken flocks in CAF

Mixed infections

After cloning, sequences of the clones were compared to the original electropherograms of the (uncloned) sample showing signs of mixed infection. The electropherogram of the original sample and the clones matched in all cases, confirming in two independent sequencing experiments that the mixed infections were not due to contamination during the amplification steps.

Table 2 Virus prevalence in flocks from Central African Republic

Year	Virus prevalence (%)					Total
	0	> 0 and ≤ 25	> 25 and ≤ 50	> 50 and ≤ 75	> 75 and ≤ 100	
2008	13	2	4	3	2	24
2009	41	6	3	4	6	60
2010	51	6	0	1	2	60
Total	105	14	7	8	10	144

Virus prevalence is expressed as the number of flocks with a virus prevalence included in a percentage range.

In Central African Republic, 6 flocks out of 20 from which CAV sequences were obtained, contained strains of 2 different genotypes (Table 3), either CAF1 and CAF3, or CAF1 and CAF4 strains. CAF1 and CAF4 strains were also both found in 2 markets. In addition, analyses of VP1 clones from samples with ambiguous nucleotides revealed mixed infections in 6 individual samples with the above combinations of genotypes (CAF1 and CAF3, CAF1 and CAF4) as well as CAF2 and CAF3 (Figure 3). In 3 of these cases, both parental strains were also found in other samples from the same farm or market (Table 3).

Similarly, CAV VP1 sequences in 13 samples from Cameroon out of 35 showing ambiguous nucleotides were cloned. Analyses of clones showed that these samples contained more than one CAV genotype,

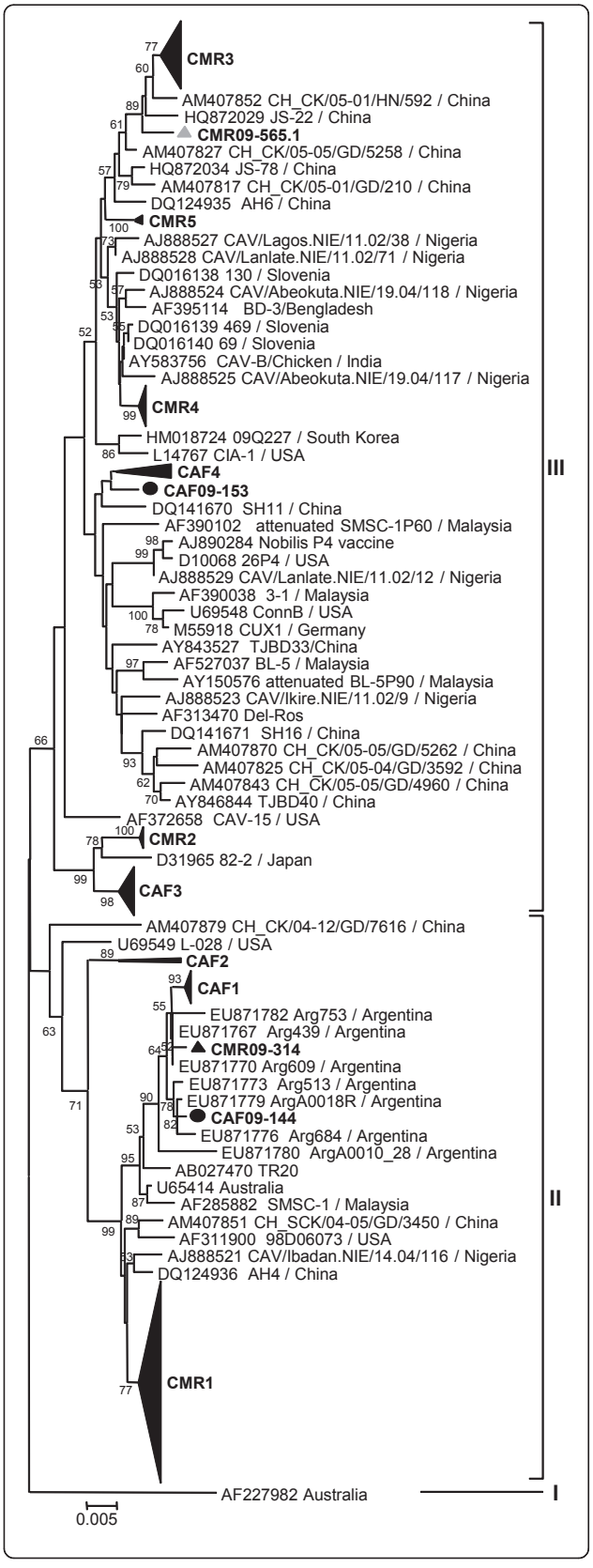

Figure 2 Phylogenetic analysis of partial VP1 sequences (1281 nucleotides) of 228 CAV strains from Central African Republic (●) and Cameroon (▲). Sequences from mixed infected samples are represented by the grey symbols. CAF and CMR clusters are shown as aggregated clusters (CAF1-CAF4; CMR1-CMR5). Only bootstrap values higher than 50% are shown.

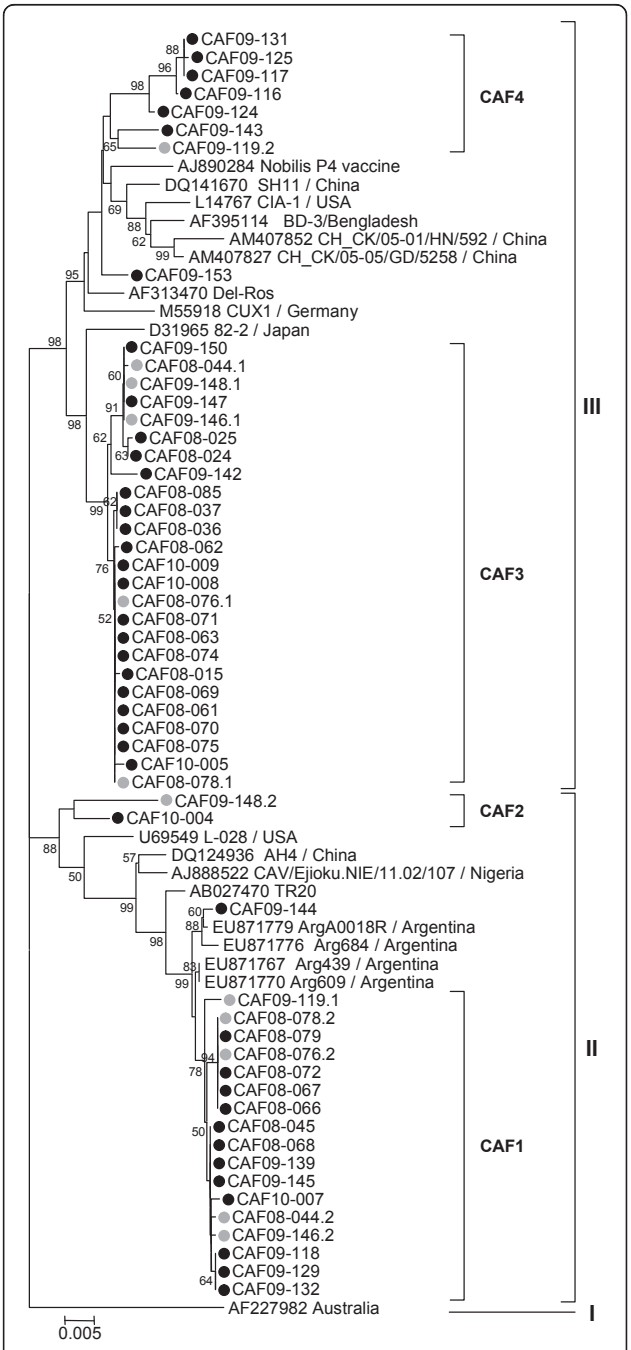

Figure 3 Detailed phylogenetic analyses of all strains from CAF (53 strains). Fewer reference strains from GenBank were used in comparison to Figure 2 due to figure size restrictions. Symbols are as in Figure 2. Only bootstrap values ≥ 50% are shown.

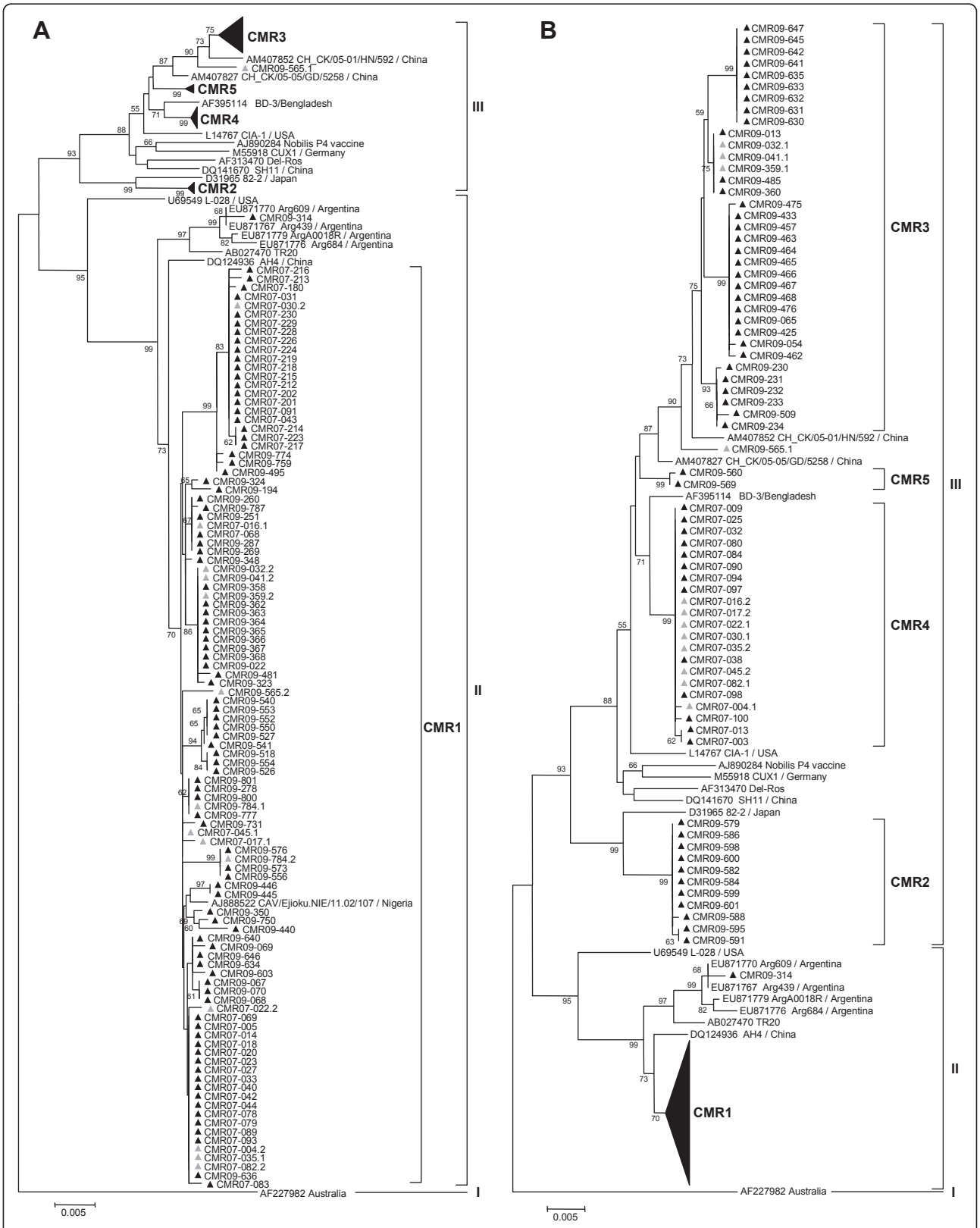

Figure 4 Detailed phylogenetic analyses of CMR1 (A) and CMR 2 to 5 (B) strains from Cameroon (175 strains). Fewer reference strains from GenBank were used in comparison to Figure 2 due to figure size restrictions. Symbols are as in Figure 2. Only bootstrap values ≥ 50% are shown.

Table 3 Mixed infections in flocks, markets and in individual samples from Central African Republic

Flock	Sample	Clusters
Flock 1	CAF08-044	CAF1 + CAF3
	CAF08-045	CAF1
Flock 2	CAF08-068	CAF1
	CAF08-069	CAF3
	CAF08-070	CAF3
	CAF08-071	CAF3
Flock 3	CAF08-072	CAF1
	CAF08-074	CAF3
	CAF08-075	CAF3
Flock 4	CAF08-076	CAF1 + CAF3
	CAF08-078	CAF1 + CAF3
	CAF08-079	CAF1
Flock 5	CAF09-131	CAF4
	CAF09-132	CAF1
Flock 6	CAF09-144	-
	CAF09-145	CAF1
	CAF09-146	CAF1 + CAF3
	CAF09-147	CAF3
Market 1	CAF09-119	CAF1 + CAF4
	CAF09-116	CAF4
	CAF09-117	CAF4
	CAF09-118	CAF1
Market 2	CAF09-142	CAF1
	CAF09-143	CAF4
Market 3	CAF09-148	CAF2 + CAF3

suggesting mixed infections in 2007 with CMR1 and CMR4 and in 2009 with CMR1 and CMR3 strains or with 2 distinct CMR1 strains (CMR09-784.1 and CMR09-784.2) differing by 7 nucleotides (Figure 4).

Phylogenetic analyses of amino acid sequences

At the amino acid level, the groups I to III defined at the nucleotide level were less clearly distinguishable (Figure 5). Nevertheless most of the CAF/CMR strains still clustered with sequences of their own nucleotide sequence group sharing specific amino acid sequences (Table 4), except for CMR09-440 (CMR1) and CMR09-630 (CMR3), both of which clustered together with an Argentinian strain (ArgA0010_28), as a result of Q139K and Q144E mutations located in the hypervariable region. CAF2 and CAF4 sequences, CAF3 and CMR2, and CMR3 and CMR5 sequences were identical at the amino acid level as a result of synonymous mutations (Table 4). All CAF/CMR clusters shared amino acids with published

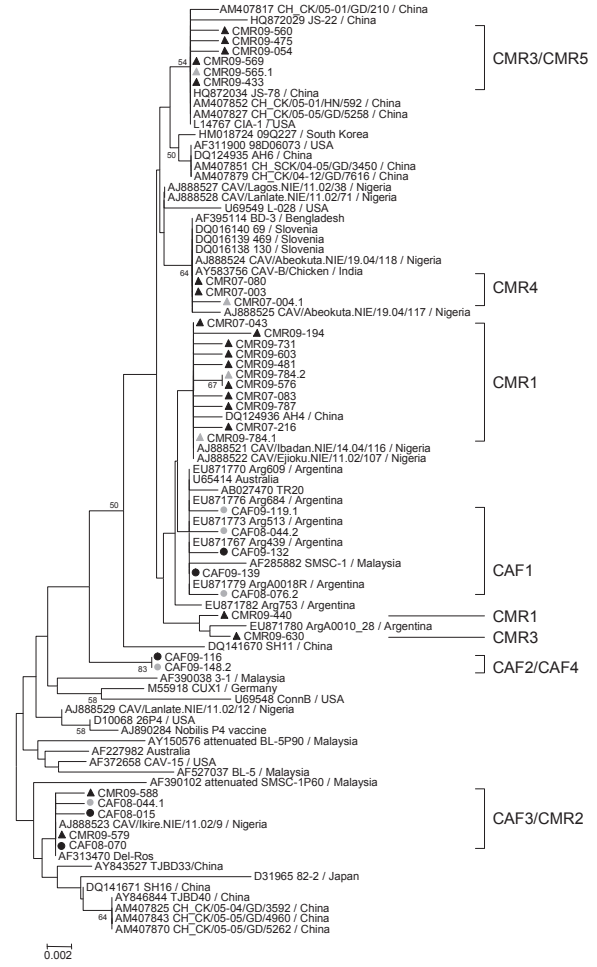

Figure 5 Phylogenetic analysis of the predicted amino acid sequences. Representative strains from each CAF and CMR clusters were used and all strains with amino acid substitutions compared to a reference strain within the group were included. Symbols are as in Figure 2. Only bootstrap values ≥ 50% are shown.

sequences except the CAF2/CAF4 group that had a unique amino acid pattern (Table 4, Figure 5).

Sixteen unique amino acid substitutions were observed in the strains from Central African Republic and Cameroon (R6L, R9K, F77L, G74E, S178P, G219E, S229Y, I285V, S300N, M311I, K341R, Q351H, T361A, D366E,

Table 4 Amino acid patterns in VP1 protein of the African genomic groups

Cluster	22	75	97	139	144	287	290	370	413
CAF1	H	I	L	Q	Q	T	P	S	A
CAF2/CAF4	H	V	M	Q	Q	S	A	G	S
CAF3/CMR2	H	V	M	K	E	S	A	G	S
CMR1	H	I	L	Q/K	Q/E	T	A	S	A
CMR3/CMR5	N	I	L	Q/K	Q/E	A	A	S	A
CMR4	H	I	L	Q	Q	A	A	T	A

G398D, G426S) and 3 substitutions were found in 3 to 6 strains (L190M, T215A, V385I).

Discussion

The overall flock seroprevalence of 36.7% in CAF appeared to be lower than in most other developing countries. In Nigeria, the only African country from where such data are available, a flock seroprevalence of 55% was reported in 20 flocks including broilers, pullets, cockerels and layers in the main hub of the poultry industry in the Southwest of the country [27]. Even a seroprevalence of 89% was reported later in chicken flocks of 4 to 12 months of age [24]. Also countries such as China [28], Malaysia [29], Japan [4], India [30] and Hungary [31] reported flock seroprevalences ranging from 66 to 100%. Normally the seroconversion is not homogeneous within a flock and tends to increase with age, complicating comparisons between studies [5,32]. For instance broiler flocks of 3 to 10 weeks have a similar seroprevalence in Nigeria (40%) [27] and in CAF (34%) and the seroprevalence in the flocks ranges from 20 to 100% in both countries (Figure 1A).

As in Nigeria, chickens are not vaccinated against CAV in Central African Republic. All sampled flocks except 2, were older than 2 to 3 weeks, the age at which the maternal antibodies wane [5,19,33]. Thus the antibodies detected are likely the result of a field infection and are not maternally derived nor vaccine induced antibodies.

Similar overall virus prevalences were found in 2007 and 2009 in Cameroon (39% and 34.9%) and in 2008 in Central African Republic (34.2%). In 2009 and 2010, the prevalence decreased to 14.3% and 10.4% in Central African Republic. This trend to lower incidence rates in Central African Republic may correspond to an improved sensitivity to hygiene due to farmer awareness in the aftermath of the first results of the study. The above values were also surprisingly low compared to other countries such as China (87%) [34], Malaysia (80%) [29] and Argentina (49%) [35] but comparable to Nigeria (41%) [23] for the 2007–2008 period. Although the prevalence may vary with time, this may indicate that the virus is less prevalent in sub-Saharan Africa than in Asia or South America. Nevertheless, prevalence rates of viral nucleic acids and antibodies may be underestimated in particular when the prevalence is low in a flock and/or low numbers of chickens were analyzed per flock. For instance, by analyzing 4 samples per flock, the certainty of detecting antigens or antibodies in a infected flock decreases from 93% when the prevalence is 50%, to 68% when the prevalence is 25%. A lower expected virus prevalence in the digestive tract compared to lymphoid organs [29], the relative young age of the animals, and possibly co-infections with other pathogens may also contribute to underestimate prevalence rates.

CAV DNA could be detected in cloacal samples of a large majority (76.9%) of the seropositive chickens, indicating that these animals may shed the virus despite antibodies (Figure 1B). While antibodies normally develop within 1 to 3 weeks [6,8,12,36], viral particles can be detected in feces up to 5 to 7 weeks post infection [8,12] suggesting that the virus is cleared with considerable delay after the development of virus-neutralizing antibodies. If we assume that most chickens become infected early after the waning of maternal antibodies, persistence of virus may even be longer. This could explain the high prevalence of virus in seroconverted animals. Alternatively co-infections with very virulent IBDV, which is also circulating in West and Central Africa [37-39], may cause a delay in development of anti-CAV antibodies, resulting in CAV persistence and a prolonged viral shedding in feces [20]. Besides the detection of a second round of viral infection cannot be ruled out although the expected excretion period would be short [12].

Although there is no clear geographic clustering of CAV strains worldwide and despite the low mutation rate at the amino acid level [21], most of the strains sequenced in CAF and CMR belonged to 9 distinct clusters at the nucleotide level and were not intermixed with strains from other continent. This suggests multiple introductions of CAV in Central Africa resulting in several clusters that emerged locally and had time to spread and diverge. Normally, chicks used to populate commercial farms in CAF are imported from Europe, but European strains (Germany, Slovenia) available in GenBank are phylogenetically not the closest relatives.

In our study, 6 flocks contained more than 1 CAV strain and 19 samples revealed mixed infections as confirmed by cloning. Similarly, cloning of VP1 sequences from Nigeria [23] and the USA [40] revealed the presence of 2 different strains (belonging to groups II and III) in the same sample. Sequences exhibiting ambiguous nucleotides have also been found in China [34] and Brazil [41] but these were not further investigated. Thus, mixed infection by CAV strains may be a relatively common event, but its impact is currently unknown.

Conclusions

Despite the low (sero) prevalence found in Central African Republic and Cameroon compared to other countries, several clusters including only African strains were found. This suggests multiple introductions of CAV that spread and diverged locally. Mixed genotype infections together with the observation of CAV DNA in cloacal samples despite antibodies suggest a suboptimal protection by antibodies or virus persistence.

Methods

Sample cohorts

In the framework of enhanced laboratory surveillance for avian viruses in the aftermath of avian influenza outbreaks in West and Central Africa, an average of 4 (between 1 and 16) cloacal samples per flock (broilers or layers) in 5 randomly selected farms were collected every month between June 2008 and December 2010 ($n = 606$) in Bangui (CAF). Cloacal swabs ($n = 198$) were also collected in two live bird markets every month between April 2009 and December 2010. In addition, 400 sera from swabbed birds were collected. Whenever recorded (in 73% of the flocks), the age of the animals ranged from 18 to 68 days for broiler flocks and from 8 weeks to 3 years for layer flocks.

In Cameroon, 287 cloacal swabs and 582 pooled tracheal-cloacal swabs from chicken were collected in 2007 (January to June) and 2009 (April to June) respectively in farms only (2007) or in farms and live bird markets (2009) within a 50 km radius around the town of Bamenda, North West Region. The age of the animals was not recorded.

Enzyme-linked immunosorbent assay (ELISA)

A commercial competitive ELISA kit (FlockCheck® CAV, IDEXX, Hoofddorp, The Netherlands) was used to detect specific antibodies against CAV in chicken sera ($n = 400$) at a 1:10 dilution. Sera samples were collected from birds in farms ($n = 302$) and live bird markets ($n = 98$). Optical density was measured using a Multiskan Ascent reader (Thermo Labsystems, Helsinki, Finland) at 650 nm. Sample to negative (S/N) ratios were calculated for each sample and samples with S/N ratios ≤ 0.60 were considered positive.

Nucleic acid extraction, polymerase chain reactions

All swabs were discharged in 500 μl of virus transport medium (VTM) containing 2000 U/ml penicillin, 200 mg/ml streptomycin, 2000 U/ml polymyxin B, 250 mg/ml gentamycin, 60 mg/ml ofloxacin, 200 mg/ml sulfamethoxazole and 2.5 mg/ml amphotericin B. Nucleic acids were extracted from 140 μl of VTM using QIAamp Viral RNA Mini Kit (Qiagen, Venlo, The Netherlands). Extracted nucleic acids were screened for CAV DNA in a nested PCR format using previously published primers [11]. The equivalent of 2.5 μl of nucleic acids and 0.5 μl of first round PCR products were used in the first round and nested PCRs respectively. PCR reactions were carried out using the following cycling conditions: initial denaturation at 95°C for 5 min, 40 cycles of amplification at 95°C for 30 s, 54°C (1st round) or 60°C (nested) for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were visualized by gel electrophoresis.

The certainty C of detecting CAV in an infected flock for various flock sizes N and various prevalences P was calculated according to the formula of Cannon and Roe (1982, cited by [42]): $n = \left\{1 - (1 - \mathrm{C})^{1/\mathrm{PxNxsens}}\right\} \{\mathrm{N} - 0.5(\mathrm{PxN} - 1)\}$ assuming an average number of $n = 4$ samples collected per flock and a test sensitivity (sens) of 100%.

Sequencing

A fragment containing the entire VP1 coding region (nucleotides 1 to 1350) of the positive samples was amplified as 3 partially overlapping fragments with a total length of 1389 bp (from nucleotide −33 to nucleotide 1355) using several primer combinations [23]. Sequencing of purified PCR products was performed as previously described [34]. Sequence assembly and analyses were performed using SeqScape version 2.5 (Applied Biosystems, Nieuwerkerk, The Netherlands) and BioEdit [43]. The 5' end of the VP1 coding region (69 nucleotides) was not reliably sequenced for a few strains. Therefore a shorter fragment (1281 nucleotides) was used in the phylogenetic analyses in order to include as many strains as possible.

Cloning

Samples exhibiting ambiguous determination of nucleotides at various positions caused by clear double peaks in the electropherogram were further analyzed. The VP1 fragments of 1370 bp were generated starting from the original DNA sample using primers OS1F and S3R7 (1st round PCR; nucleotides −117 to 1355) and S1F and S3R1 (5'-CCCAGTACATSGTGCTGTT-3') primers (nested PCR; nucleotides −33 to 1336), purified and cloned using the TOPO TA cloning kit (Life Technologies, Merelbeke, Belgium) as described previously [23]. Ten to 24 colonies were selected and inserts were sequenced with M13 primers (Life Technologies). To exclude the possibility of crosscontamination, long amplicons (1370 bp) suitable for cloning were generated twice in two independent experiments (on different days) and were cloned separately (again on different days) for two samples and similar results were obtained. Both experiments gave clones of the same sequences although with different frequencies.

Phylogenetic analyses

Genetic distances were calculated with MEGA v5.03 [44] according to the Kimura 2-parameters model. Phylogenetic relationships were inferred by comparing the African strains with all CAV DNA sequences available on GenBank (downloaded in February 2012) after removal of short sequences and sequences with insertions or deletions resulting in frame shifts. Datasets were aligned using ClustalW [45]. Trees were calculated with the Neighbour-Joining method, using the Kimura 2-parameters model and

1000 bootstrap replicates for the nucleotide tree and with the Poisson model for the amino acid tree (MEGA v5.03; data not shown). Reference strains from GenBank were selected based on these preliminary analyses. Trees including the selected reference strains from GenBank and all (Figure 2) or a subset (Figure 5) of the sequences generated in this study were calculated using the same parameters as described above. Fewer representative strains from GenBank were used in Figures 3 and 4 due to figure size restrictions. All gene sequences can be found under accession numbers [EMBL: HE662876 to HE663056 and HE686970 to HE687016]. Strains were designated using the following nomenclature: 3-letter country code (CAF = Central African Republic; CMR = Cameroon)_year-sample number (last 3 digits). Sequences from mixed infected samples are named by adding .1 or .2 after the strain name.

Abbreviations

bp: Base pair(s); CAF: Central African Republic; CAV: Chicken anemia virus; CMR: Cameroon; ELISA: Enzyme-linked immunosorbent assay; IBDV: Infectious bursal disease virus; mKd: Maximal Kimura distance; PCR: Polymerase chain reaction; S/N: Sample to negative; VP1: Viral protein 1; VTM: Virus transport medium.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

CJS, EN, MPO, AL and CPM designed the study. CJS, GFK and BPM carried out the experiments and analyzed the results. CJS drafted the manuscript and AL, EN, MPO and CPM contributed to the discussion and reviewed the manuscript. All authors read and approved the final manuscript.

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Résumé:

La viande de volaille et les œufs constituent une source de protéines bon marché mais la production avicole est menacée par deux maladies virales, la grippe aviaire hautement pathogène et la maladie de Newcastle, ayant des implications économiques et de santé publique à travers le monde. L'introduction du virus de l'influenza aviaire (AIV) hautement pathogène H5N1 en Afrique en 2006 a souligné la nécessité d'une meilleure compréhension d'AIV en Afrique. Grâce à des études de surveillance, nous avons constaté que le virus H5N1 ne circulait plus après 2008 en Afrique subsaharienne. Toutefois, les analyses phylogénétiques réalisées sur le génome de virus faiblement pathogènes H5N2 trouvés chez des oiseaux sauvages au Nigeria ont révélé des caractéristiques de virus réassortants. La similitude d'un gène avec ceux trouvés dans d'autres virus d'Afrique australe renforce l'idée qu'AIV est capable de persister et circuler en Afrique. Nous avons également montré que de nouvelles souches virulentes du virus de la maladie de Newcastle (NDV) constituent la majorité des souches détectées. Leur distance génétique par rapport aux autres souches de NDV connues, leur diversité génétique et leur dispersion géographique suggèrent que ces souches ont probablement évolué localement, circulent depuis un certain temps dans la région et que le commerce et le mouvement d'animaux ont contribué à leur propagation. Nos résultats suggèrent également que la contribution des oiseaux sauvages à la dispersion des souches virulentes du NDV est probablement limitée. Au Luxembourg cependant, les oiseaux sauvages pourraient être un acteur important pour l'introduction du NDV.

Mots clés: virus de l'influenza aviaire, virus de la maladie de Newcastle, épidémiologie moléculaire, évolution, surveillance

Abstract:

Poultry meat and eggs constitute one of the cheap sources of protein around the world but poultry production is threatened by two main viral diseases, highly pathogenic avian influenza and Newcastle disease, with economic and public health implications worldwide. The introduction of highly pathogenic avian influenza H5N1 virus in Africa in 2006 highlighted the necessity of a better understanding of avian influenza virus (AIV) in Africa. Through surveillance studies, we found that H5N1 virus was not circulating anymore in sub-Saharan Africa after 2008. However, phylogenetic analyses performed on the genome of low pathogenic H5N2 viruses found in wild birds in Nigeria revealed that they were reassortants. The similarity of one gene to those found in other AIV viruses from Southern Africa strengthened the hypothesis that AIV may actually persist and circulate in Africa. We have shown that new virulent strains of Newcastle disease virus (NDV) constituted the majority of the strains detected. Their genetic distance compared to other NDV strains, their genetic diversity and their geographic dispersion in West and Central Africa suggested that these strains probably evolved locally, that they circulated for some time in the region and that trade and movement of animals likely contributed to their spread. Our findings also suggested that the contribution of wild birds to the dispersion of virulent strains of NDV was probably limited. In Luxembourg however, wild birds may be an important player for the introduction of NDV strains.

Keywords: avian influenza virus, Newcastle disease virus, molecular epidemiology, evolution, surveillance