Phylogeny and genotyping of recent avian low-pathogenic H5 subtype influenza viruses from French ducks

F.-X. Briand, G. Le Gall-Reculé, C. Guillou-Cloarec, K. Ogor and V. Jestin

AFSSA, French Reference Laboratory for Avian Influenza and Newcastle Disease, Avian and Rabbit Virology, Immunology and Parasitology Unit, BP 53, 22440 Ploufragan, France

H5 low-pathogenic avian influenza virus (LPAIV) has the potential to become highly pathogenic and to cause serious problems in animal and public health. AIV surveillance and characterization in both wild and domestic species is therefore necessary. In order to acquire molecular information and to identify possible reassortments in French viruses, we analysed the entire genome of five H5N3, three H5N2 and two H5N1 LPAIV, isolated in France between 2002 and 2008 mostly from captive ducks (free-range commercial poultry or decoy ducks). Some of the genome sequences showed atypical characteristics, such as an insertion of 1 aa in the PB1 protein of one H5N3, a highly truncated PB1-F2 protein (11 aa in length instead of 90 aa) in one H5N2, and an insertion of 8 aa in the NS1 protein of H5N1. These two last molecular characteristics have not been described previously. Phylogenetic analysis demonstrated that all genes of French LPAIV, except the closely related matrix protein genes, clustered within the Eurasian avian influenza virus lineage and fell into at least two phylogenetic subgroups. In addition, the French H5 LPAIV were segregated into eight genotypes, suggesting that many reassortment events have occurred in H5 LPAIV in Europe. However, it is not known whether the reassortment events have occurred in wild waterfowl and/or in captive birds in direct or indirect contact with wild birds.

INTRODUCTION

Aquatic birds are the primary reservoir of type A influenza viruses. These avian influenza viruses (AIV) are classified into subtypes based on antigenic differences in their surface glycoproteins (haemagglutinin, HA; and neuraminidase, NA). So far, sixteen haemagglutinin (H1–H16) and nine neuraminidase (N1–N9) subtypes have been reported (Alexander, 2000; Fouchier et al., 2007). The AIV genome is segmented into eight negative strands, permitting the occurrence of reassortment events. Each segment contains either one gene (PB2, PA, HA, NP, NA) or two genes (PB1/PB1-F2, M1/M2, NS1/NS2) (Chen et al., 2001). Two main lineages (American and Eurasian) have evolved independently, due to limited contacts between birds from the old and new continents (Ito et al., 1995; Kawaoka et al., 1988; Lin et al., 1994; Okazaki et al., 2000; Webster et al., 1992). Exchanges of AIV genes between these two lineages are, in consequence, very rare (Krauss et al., 2007). Avian influenza viruses can evolve genetically via mutations (substitutions, insertions, deletions) and reassortments (Domingo et al., 2001; Webster et al., 1992). Mutations are due to mistakes generated by the polymerase complex during replication. Some insertions, such as the addition of polybasic amino acids at the cleavage site of H5 or H7 low-pathogenic (LP) AIV, allow the virus to become highly pathogenic (De Marco et al., 2005; Donatelli et al., 2001). This mechanism was reported for Italian H5N2 in 1997, for Italian H7N1 in 1999, for Dutch H7N7 in 2003, for South African H5N2 and for the current ‘Asian’ H5N1. Deletions in the neuraminidase have been observed as an adaptation of AIV to terrestrial poultry (Matrosovich et al., 1999). A further evolutionary phenomenon of AIV is its capacity to undergo reassortment. Co-infection of the same cell of a given host by at least two different viruses can result in mixed segments from both parental viruses. This phenomenon has given rise to pandemic viruses (Chen et al., 2009; Kawaoka et al., 1989). Poultry infection with H5 or H7 LPAIV can be asymptomatic or produce a wide range of signs, varying from mild respiratory disorders to more severe disease in the case of aggravating circumstances such as concomitant infection with other pathogens (Alexander, 2000). Because these viruses can become highly pathogenic, the presence of H5 or H7 LPAIV in poultry is notifiable and therefore regulated (European Commission, 2006). In order to further our understanding of AIV evolution and the consequences of new mutations, LPAIV H5 and LPAIV H7 from both wild and domestic birds need to be monitored and characterized. Although the characteristics

The GenBank/EMBL/DDBJ accession numbers for the new virus sequences determined in this work are CY046117–CY046186.

A supplementary figure with details of the phylogenetic analysis of the genes is available with the online version of this paper.
of asian H5 LPAIV have been previously described (Duan et al., 2007), the genetic characteristics of H5 LPAIV from European domestic ducks have been little documented, even though serological surveys have shown these species (Pekin, Muscovy and the mule hybrid) to be frequently infected by H5 viruses in Europe (Hesterberg et al., 2008). Some characteristics, based on partial sequencing and antigenic study of four H5 LPAIV from domestic ducks, have already been reported (Cherbonnel et al., 2007). We have taken this study further by analysing 10 whole genomes of the H5 subtype of LPAIV isolated in France from decoy ducks (two isolates), commercial ducks (seven isolates), and chickens (one isolate) for 7 years, between 2002 and 2008. Our aim was (i) to determine the relationships between each RNA segment and its counterpart from earlier Eurasian isolates, (ii) to assess the range of genotype diversity, (iii) to determine whether specific gene combinations might be favoured, and (iv) to identify the particular molecular traits associated with each segment.

**RESULTS**

All H5 LPAIV described in this study (Table 1) were isolated as described in Methods and their intravenous pathogenicity index (IVPI) was determined. This resulted in minimal values of 0.0 for all of them, which confirmed their low pathogenicity, in agreement with the motifs of their HA cleavage site exhibiting no multiple basic amino acids such as K and R (Table 2).

**Phylogenetic analysis**

All sequences, irrespective of the segment, belonged to the avian Eurasian lineage. The origin of each segment of the 10 French AIV is described in detail in Table 3. For each gene segment the percentages of nucleotide identities between French isolates are given in Table 4. The maximum identity was ≥ 99% for all genes except N1 and N2 (94.8% and 91.7%, respectively). Symmetrically, the minimum identity ranged between 89.5% and 95.1%, except for NS sequences (62%), owing to the presence of alleles A and B.

Phylogenetic analysis of the PB2 gene (see Supplementary Fig. S1b, available in JGV Online) showed that 9 of the 10 French AIV were closely related to the H7N1 high-pathogenicity (HP) AIV and LPAIV isolated in Italy during the outbreaks that occurred in 1999 and 2000. The only French strain that did not belong to this group was 03426, in which the PB2 gene clustered with the Italian H5N2 LPAIV isolated during the 1997 outbreak.

Phylogenetic analysis of the PB1 gene of the French AIV exhibited greater diversity, since three clusters were identified (Fig. 1a). The first cluster grouped half of the French AIV with A/chicken/Netherlands/1/03 HPAIV H7N7 and the Italian H7N3 viruses isolated in 2003. The second cluster grouped three French isolates and LP H5N2 A/duck/Mongolia/54/2001. Finally, the two other French PB1 genes were closely related to Asian viruses such as A/chicken/Nanchang/7-010/2000 H3N6.

For the PA gene (Supplementary Fig. S1b), eight French strains clustered with A/poultry/Italy/330/97 HPAIV H5N2. The two other French isolates were different. The PA genes of 05057b were closely related to Italian H5N2, whereas the PA of 03426 was grouped with a German H9N2 strain isolated in 1995 and with an H14N5 virus from Astrakhan isolated in 1982.

Phylogenetic analysis of the French H5 sequences revealed the existence of four subgroups (Fig. 1b). The first gathered 7 out of 10 H5 genes belonging to the French viruses isolated from ducks since 2006. These were closely related to A/duck/Denmark/65047/04 LPAIV (H5N2). The second subgroup contained two French viruses (05066b and 03426), a contemporary Italian isolate A/turkey/Italy/1258/05 LPAIV (H5N2) and A/duck/Mongolia/54/01 LPAIV (H5N2). According to current HA phylogeny, the gene from 02166 did not belong to any of the previous subgroups, but according to the arbitrary cut-off conditions (explained in Methods), formed a group with a single French virus A/duck/France/05066a/2005 H5N2 (Cherbonnel et al., 2007).

For the NP segment (Supplementary Fig. S1c), three sublineages were identified in the French strains. The first group contained 06964, 061054, 070090b, 080036 and

**Table 1. French H5 LPAIV isolates used in this study**

<table>
<thead>
<tr>
<th>Reference viruses</th>
<th>Shortened name</th>
<th>Host</th>
<th>Isolation year</th>
<th>French location</th>
<th>Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/duck/France/02166/2002</td>
<td>02166</td>
<td>Commercial duck</td>
<td>2002</td>
<td>North-west</td>
<td>H5N3</td>
</tr>
<tr>
<td>A/chicken/France/03426/2003</td>
<td>03426</td>
<td>Chicken</td>
<td>2003</td>
<td>West</td>
<td>H5N2</td>
</tr>
<tr>
<td>A/duck/France/05057b/2005</td>
<td>05057b</td>
<td>Commercial mule duck</td>
<td>2005</td>
<td>West</td>
<td>H5N2</td>
</tr>
<tr>
<td>A/duck/France/05066b/2005</td>
<td>05066b</td>
<td>Commercial mule duck</td>
<td>2005</td>
<td>West</td>
<td>H5N1</td>
</tr>
<tr>
<td>A/duck/France/06436/2006</td>
<td>06436</td>
<td>Commercial mule duck</td>
<td>2006</td>
<td>South-west</td>
<td>H5N3</td>
</tr>
<tr>
<td>A/mallard/France/06964/2006</td>
<td>06964</td>
<td>Decoy mallard</td>
<td>2006</td>
<td>West</td>
<td>H5N3</td>
</tr>
<tr>
<td>A/mallard/France/061054/2006</td>
<td>061054</td>
<td>Decoy mallard</td>
<td>2006</td>
<td>North</td>
<td>H5N3</td>
</tr>
<tr>
<td>A/Muscovy duck/France/070090b/2007</td>
<td>070090b</td>
<td>Breeder Muscovy duck</td>
<td>2007</td>
<td>North-west</td>
<td>H5N3</td>
</tr>
<tr>
<td>A/duck/France/080032/2008</td>
<td>080032</td>
<td>Commercial mule duck</td>
<td>2008</td>
<td>West</td>
<td>H5N2</td>
</tr>
<tr>
<td>A/duck/France/080036/2008</td>
<td>080036</td>
<td>Commercial duck</td>
<td>2008</td>
<td>West</td>
<td>H5N2</td>
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</tbody>
</table>
The second consisted of 02166, 05066b, 06436, 080032 and LPAIV H7N3 A/mallard/Netherlands/12/2000. The third was represented by 05057b and LPAIV H5N2 A/teal/Italy/3951-28/05. According to the phylogenetic tree, 03426 did not belong to any group; the closest displayed 94% identity.

Three different NA subtypes were identified in the 10 French H5 LPAIV studied (five N3, three N2 and two N1). The two French N1 sequences belonged to the same lineage and were closely related to all the Italian LPAIV and HPAIV H7N1 detected between 1999 and 2000 (Supplementary Fig. S1d). Of the three N2 sequences (Supplementary Fig. S1e), that of 080032 was closely related to LPAIV H5N2 A/duck/Denmark/65047/04 and H6N2 A/Bewick swan/Netherlands/1/2005. The N2 gene of 03426 had a common ancestor with the Italian HPAIV H5N2 isolated in 1997 whereas the N2 gene of 05057b did not belong to any group. All French and prototype N3 genes studied here (Supplementary Fig. S1f) were shown to be closely related within the same subgroup, this corresponding to the most recent N3 gene.

No major sublineage has yet been described for the M gene phylogeny of AIV, although some small groups showed a significant bootstrap value (Supplementary Fig. S1g). For instance, the M genes of 02166 and 080032 grouped with those of other French strains (A/duck/France/05054a/2005 and A/duck/France/05056a/2005; Cherbonnel et al., 2007), whereas the M genes of 05066b and 080036 formed another cluster. The M gene of 05057b was closely related to its counterpart from LPAIV H5N2 A/Denmark/65047/04, unlike the M genes of 06964 and 061054, which were grouped together.

For the NS gene, two major groups were apparent (Fig. 1c), corresponding to the A and B alleles, as already described (Scholtissek & von Hoyningen-Huene, 1980; Treanor et al., 1989).

### Table 2. Genetic traits of French H5 LPAIV isolated between 2002 and 2008

<table>
<thead>
<tr>
<th>Reference virus</th>
<th>Cleavage site IVPI</th>
<th>PB2 (E627K)</th>
<th>PB1-F2 length (aa)</th>
<th>NA deletion</th>
<th>PB1 length (aa)</th>
<th>NS1 allele</th>
<th>NA (N2 numbering)</th>
<th>N2 numbering</th>
</tr>
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<tbody>
<tr>
<td>02166</td>
<td>0.0</td>
<td>E</td>
<td>D</td>
<td>D</td>
<td>758</td>
<td>90</td>
<td>I</td>
<td>238</td>
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<td>03436</td>
<td>0.0</td>
<td>E</td>
<td>D</td>
<td>D</td>
<td>757</td>
<td>90</td>
<td>I</td>
<td>230</td>
</tr>
<tr>
<td>05066b</td>
<td>0.0</td>
<td>E</td>
<td>D</td>
<td>D</td>
<td>757</td>
<td>90</td>
<td>I</td>
<td>230</td>
</tr>
<tr>
<td>06436</td>
<td>0.0</td>
<td>E</td>
<td>D</td>
<td>D</td>
<td>757</td>
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<td>I</td>
<td>230</td>
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<td>E</td>
<td>D</td>
<td>D</td>
<td>758</td>
<td>90</td>
<td>I</td>
<td>230</td>
</tr>
<tr>
<td>061054</td>
<td>0.0</td>
<td>E</td>
<td>D</td>
<td>D</td>
<td>758</td>
<td>90</td>
<td>I</td>
<td>230</td>
</tr>
<tr>
<td>070090b</td>
<td>0.0</td>
<td>E</td>
<td>D</td>
<td>D</td>
<td>757</td>
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<td>I</td>
<td>230</td>
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<td>758</td>
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<td>080036</td>
<td>0.0</td>
<td>E</td>
<td>D</td>
<td>D</td>
<td>758</td>
<td>90</td>
<td>I</td>
<td>230</td>
</tr>
</tbody>
</table>

HPAIV H7N7 A/chicken/Netherlands/1/03. The second consisted of 02166, 05066b, 06436, 080032 and LPAIV H7N3 A/mallard/Netherlands/12/2000. The third was represented by 05057b and LPAIV H5N2 A/teal/Italy/3951-28/05. According to the phylogenetic tree, 03426 did not belong to any group; the closest displayed 94% identity.

The original reassortment events that resulted in the French viruses were determined from a diagrammatic representation of the genotypes (Fig. 2). Only three French H5N3 viruses (06964, 061054 and 070090b) displayed the same genotype. No French genotype was identical to the Eurasian reference viruses selected for this study although a few of their genes were very closely related to French viruses. Each French virus often had several segments of different origin. For example, 06436 and 080032 were composed of at least six genes from different subgroups (Table 3). Each gene from the French and reference Eurasian viruses (within the limit of the viruses studied) appeared, at first sight, to show a random distribution, as already observed for AIV (Campitelli et al., 2008). When only the French strains were considered, and except for the H5N2 isolated from chicken, they all contained a PB2
segment from the A/chicken/Italy/5093/99 subgroup. Similarly, the PA segment from the A/poultry/Italy/330/97 subgroup was present in eight French viruses but not in 03426 and 05057b. In addition, the combination of PB2 from the A/chicken/Italy/5093/99 subgroup, PB1 from the A/duck/Mongolia/54/01 subgroup and PA from the A/poultry/Italy/330/02 subgroup was present in the French virus isolated in 2002 and was again detected in the two viruses isolated in 2008. Another PB2, PB1, PA combination (with the same PB2 and PA but a PB1 from the A/chicken/Netherlands/1/2003 subgroup) was observed in the four viruses isolated in 2006 and 2007.

### Detailed molecular characterizations

The prominent genomic features are reported in Table 2. No French NA sequences showed an NA stalk deletion

<table>
<thead>
<tr>
<th>Genotype H5 isolate*</th>
<th>Lineage of gene segment†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PB2</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
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<tr>
<td>9</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

*The year of isolation is given by the first two numbers of the reference of the H5 isolate.
†Prototype strain (as defined in Methods) of the genetic group to which the French isolate is related.

### Table 4. Nucleotide sequence identity between the most and the least closely related gene segments of French influenza virus isolates

<table>
<thead>
<tr>
<th>Gene segment</th>
<th>Maximum identity (%)</th>
<th>Minimum identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB2</td>
<td>061054</td>
<td>070090b</td>
</tr>
<tr>
<td>PB1</td>
<td>06964</td>
<td>070090b</td>
</tr>
<tr>
<td>PA</td>
<td>070090b</td>
<td>080036</td>
</tr>
<tr>
<td>HA</td>
<td>06154</td>
<td>06964</td>
</tr>
<tr>
<td>NP</td>
<td>06964</td>
<td>070090b</td>
</tr>
<tr>
<td>N1</td>
<td>05066</td>
<td>080036</td>
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<tr>
<td>N2</td>
<td>03426</td>
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<tr>
<td>N3</td>
<td>06964</td>
<td>061054</td>
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<tr>
<td>M</td>
<td>05066b</td>
<td>080036</td>
</tr>
<tr>
<td>NS</td>
<td>061054</td>
<td>080032</td>
</tr>
</tbody>
</table>

*The year of isolation is given by the first two numbers of the reference of the H5 isolate.
†Prototype strain (as defined in Methods) of the genetic group to which the French isolate is related.

Fig. 1. (on following pages). Phylogenetic analyses. The trees were generated by the neighbour-joining algorithm. Only bootstrap values >75% are shown. French sequences obtained in this study are labelled with a blue circle and prototype sequences with a red circle. (a) Partial representation of the tree obtained from the phylogenetic analysis based on nucleotides 1–2271 of the PB1 gene to determine the different clades. (b) Partial representation of the tree obtained from the phylogenetic analysis based on nucleotides 17–1608 of the H5 gene to determine the different clades. (c) Partial representation of the tree obtained from the phylogenetic analysis based on nucleotides 7–818 of the NS gene to determine the different clades. The scale bars show evolutionary distances for PB1, H5 and NS.
except strain 03426, which had a deletion of 19 aa. Although the neuraminidase of this isolate was closely related to the Italian H5N2 1997 isolates (95% identity), only one of these (A/poultry/Italy/392/97) had a similar NA deletion to that of the French N2.

Four French H5 AIV isolates had a PQRETR/GLF cleavage-site motif on the HA gene, five had PQKETR/GLF, and one [070090b (H5N3)] had PQKEAR/GLF. Several amino acids reported to be involved in H5N1 HPAIV pathogenicity in mice (Jiao et al., 2008) or to have a role in host specificity (Shaw et al., 2002) were also observed in all eight segments of the French AIV isolates. Thus a valine residue was present at position 28 of the M2 protein from 05066b and a serine residue at position 42 of the NS1 protein from six French H5 strains. None of the isolates had mutations at position 627 of PB2, allowing enhanced replication in mammalian cells (Hatta et al., 2001; Shinya et al., 2004), or position 701, which has been associated with increased pathogenicity in mice (Li et al., 2005). Moreover, on M2 and NA, no mutation conferring amantadane or oseltamivir resistance (Abed et al., 2006; Scholtissek et al., 1998) was present, except V27I in the M2 protein from 06436, which could confer amantadane resistance (Hayden, 2006) (Table 2). One atypical molecular particularity was the presence of a highly truncated PB1-F2 in the 03426 strain, which is new for AIV (Table 5). This PB1-F2 was only 11 aa long, whereas it is usually more than 78 aa long in the avian lineage, the lowest reported length being 18 aa (Zell et al., 2007). In addition, there was an insertion of a glutamine residue at the C-terminus of the PB1 protein in 02166, 080032 and 080036. This particularity has been observed at low frequency in all the Eurasian AIV (16.25%; 160/985). Finally, the most atypical molecular characteristic was probably the addition of 8 aa at the C-terminus of the 05066b NS1 protein.

**DISCUSSION**

Full sequencing and specific sequence analysis of 10 French H5 LPAIV genomes, obtained from virus strains isolated between 2002 and 2008, allowed the identification of genetic reassortment events and particular genetic traits. To our knowledge, this is the first detailed study of H5 LPAIV from European domestic ducks. Several approaches have been described for genotyping and investigating the reassortment phenomenon in influenza virus. However, some previous studies produced different subgroups for each segment without synthesizing data to consider the constellation of the eight genes for each strain (Campitelli et al., 2008; Hatchette et al., 2004). Such an approach could not be used to identify combinations between segments as each segment was treated independently. Only a few authors (Cheung et al., 2007; Duan et al., 2007; Xu et al., 2007) have defined prototype viruses as gene donors to identify different genotypes. However, these authors used a group of strains, referred to under the generic term ‘virus strain from aquatic bird’ or ‘H9N2’, but not individual strains, to describe their prototype viruses. Their approach may hide reassortment events between ‘aquatic bird’ and other subgroups. Although our method was derived from the latter, we identified unique viruses as Eurasian prototype viruses.

We thus defined 11 prototype viruses after choosing the closest viruses to the French H5 LPAIV, which possessed at least two genes in common with the French viruses. In addition, the prototype viruses selected had been isolated before or in the same year as the French viruses.

Using this approach, we identified at least two sublineages for each influenza virus segment, except M. The existence of segments from different sublineages would favour multiple combinations and generate viruses with different genotypes. Thus, the genotypes of the French H5 LPAIV did not correspond to the genotypes of prototype strains, although many genes of the French and prototype viruses had a common origin. A preliminary analysis of a more recent French LPAIV strain, A/duck/France/090045/09 (H5N3), isolated in February 2009, revealed that this virus also displayed a different genotype from the other viruses (French and Eurasian prototype viruses) studied here. Only three of the 10 French AIV (06964, 061054 and 070090b) belonged to the same genotype. These three viruses were isolated over a 4 month period (between late October 2006 and mid-February 2007) from decoy mallards in northern and western France and from breeder Muscovy ducks in western France, all rearing sites being under the same migratory pathway. This may confirm that H5 viruses can be transmitted from wild duck to domestic duck and possibly vice versa. The many reassortments described in this study confirm the high frequency of reassortment events in influenza viruses (Campitelli et al., 2008; Macken et al., 2006). This phenomenon has been shown to allow the emergence of new viruses which can then spread more efficiently (Hatchette et al., 2004). Gene reassortment has been reported to occur at random (Campitelli et al., 2008; Hatchette et al., 2004). However, although only a small number of French H5 LPAIV were involved in this study, we observed a major lineage for the PB2 and PA genes. This could be due to an adaptation to the duck host. In fact, strain 03426 isolated from chicken was the only virus not
Genetic characteristics of recent French H5 LPAIV

1997
A/poultry/Italy/330/97 H5N2

1999
A/chicken/Italy/5093/99 H7N1

2000
A/chicken/Nangchang/7-010/00 H3N6 A/mallard/Netherlands/12/00 H7N3

2001
A/duck/Mongolia/54/01 H5N2

2002
A/duck/Spain/02166/02 H5N3 A/mallard/Italy/37/02 H5N3

2003
A/chicken/France/03426/03 H5N2 A/chicken/Netherlands/1/03 H7N7

2004
A/duck/Denmark/05047/04 H5N2 A/duck/Italy/775/04 H5N3

2005
A/duck/France/05066b/05 H5N1 A/duck/France/05057b/05 H5N2 A/poultry/Italy/1258/05 H5N2 A/teal/Italy/3931-38/05 H5N2

2006
A/mallard/France/06964/06 H5N3 A/mallard/France/061054/06 H5N3 A/duck/France/06436/06 H5N3

2007
A/Muscovy duck/France/070090b/07 H5N3

2008
A/duck/France/080032k/08 H5N2 A/duck/France/080036b/08 H5N1
Table 5. Distribution of 1937 avian sequences according to the PB1-F2 length as deduced from protein available from the NCBI Influenza Virus Resource

<table>
<thead>
<tr>
<th>Avian PB1-F2 length (aa)</th>
<th>No. of sequences*</th>
<th>Percentage of sequence</th>
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</thead>
<tbody>
<tr>
<td>11</td>
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</tr>
<tr>
<td>18</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>1937</strong></td>
<td><strong>100</strong></td>
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*In this study.

Influenza virus non-structural protein 1 (NS1) is a virulence factor that inhibits the induction and/or production of interferon. Four residues located in the C-terminal domain of the NS1 protein (between amino acids 227 and 230) were also shown to constitute a potential PDZ domain ligand. PDZ domains are protein–protein recognition modules that organize diverse cell-signalling assemblies. They specifically recognize short C-terminal peptide motifs of 4–5 aa. Proteins containing PDZ domains play important roles in the transport, localization and assembly of supramolecular signalling complexes, organizing cell polarity, receptors and downstream effectors (Hale et al., 2008). Thus, truncation or modification of NS1 in this domain could have a role in pathogenicity. The implication of a deletion in the C-terminal domain of this protein has been described by Dundon et al. (2006), who noted that H7N1 HPAIV have a full-length NS1 protein of 224 aa, whereas the NS1 protein of H7N1 LPAIV is 230 aa (non-truncated) or 220 aa long (truncated). To our knowledge, the addition of amino acids has seldom been described (11 AI) in avian viruses possessing the A allele but never in those with the B allele as for the NS1 of 05066b. These extensions were also present in the human lineage and could play a role in nuclear and nucleolar localization signalling (Melen et al., 2007). This particularity of the NS1 of 05066b H5N1 LPAIV does not modify the apparent pathogenicity of this virus for mule ducks and chickens (IVPI value=0), as demonstrated by the absence of clinical signs and macroscopic lesions following experimental infection in our laboratory. However, neither microscopic study of organs nor transcriptomic analyses were carried out. The virus would have to be compared by reverse genetics with the same virus without the NS1 insertion to analyse the real impact of this mutation.

The domain of PB1-F2 contains the mitochondrial signal and can trigger apoptosis (Zell et al., 2007). Recent comparisons of avian with human influenza viruses revealed that many species-associated linked amino acid signatures are also located in the C-terminal domain of PB1-F2 (Zell et al., 2007). This demonstrates the importance of further investigating the role of PB1-F2 in interspecies infection. The truncated PB1-F2 of H5N2 03426 may have a role in adaptation to this new host. Similarly, a shorter length of PB1-F2 (11 aa instead of 90 aa) has been identified in classical swine influenza viruses (Zell et al., 2007).

It is now important to investigate the implications of these various molecular characteristics of French AIV, particularly in terms of pathogenicity and contagiousness. A reverse-genetics approach is currently being applied in our laboratory, together with in vivo experiments in high-containment facilities. In addition, it would be interesting to fully sequence more European AIV (H5 and non-H5) and identify the phylogenetic relationships between the different subtypes isolated. The genotypes and gene origins of other Eurasian viruses also need to be analysed in order to determine the extent to which viruses have similar or unique features, and also to identify all the major subgroups, not only those containing the French H5 LPAIV.

**METHODS**

**Viruses.** Ten French H5 LPAIV (five H5N3, three H5N2 and two H5N1), collected between 2002 and 2008 from chickens or from wild or domestic ducks, were characterized (Table 1). Four of these H5
viruses had already been isolated and subjected to preliminary characterization. The others were isolated and identified using the same methods. Briefly, clutches of cloacal, tracheal or oropharyngeal swab samples were inoculated into 9-day-old specific-pathogen-free (SPF) embryonated chicken eggs by the allantoic route. The eggs were incubated at 37°C for up to 5 days (with a second passage if necessary) until embryonic death. The allantoic fluid was then harvested and tested for haemagglutination activity. The AIV was identified using reference sera (OIE Manual: World Organization for Animal Health, 2008) and RT-PCR/sequencing (see below). The intravenous pathogenicity index (IVPI) was determined as follows. Fresh allantoic fluid with a HA titre >1/16 was diluted in sterile isotonic saline buffer. This diluted virus was injected intravenously (0.1 ml) into 6-week-old SPF chickens, and birds were examined at 24 h intervals for 10 days. This standard procedure is explained in the OIE Manual (World Organization for Animal Health, 2008).

**RT-PCR and sequencing.** Genomic influenza virus RNA was extracted from 200 µl allantoic fluid, using the QIAamp viral RNAeasy Mini kit (Qiagen). RNAs were reverse transcribed using Superscript II RT-PCR and sequencing. Fresh allantoic fluid with a HA titre >1/16 was diluted in sterile isotonic saline buffer. This diluted virus was injected intravenously (0.1 ml) into 6-week-old SPF chickens, and birds were examined at 24 h intervals for 10 days. This standard procedure is explained in the OIE Manual (World Organization for Animal Health, 2008).

**Phylogeny and genotyping.** Phylogenetic analyses were inferred using MEGA3.1 (Kumar et al., 2004) for the neighbour-joining analyses and confirmed by PhyML for the maximum-likelihood analyses. An initial phylogenetic analysis, using a selection of approximately 50 human, 20 swine, 3 equine and 120 avian sequences from the American and Eurasian lineages, was done to determine the membership of each gene. A more accurate phylogenetic analysis, involving about 250 Eurasian lineage complete avian sequences, was then performed for each influenza virus segment. These sequences were obtained from the representative subtypes and locations that were available in the databases. Only a few H5N1 HPAIV and Asian H9N2 were selected due to their over-representation. All sites from the prototype strains were used for the alignment. The phylogenetic trees obtained for the PB1 and H5 and NS genes in Fig. 1(a), (b) and (c) respectively, only that part of the tree containing the French strains studied is shown.

Reassortment between the studied and prototype viruses was visualized by attributing a single colour to each prototype. When several prototype viruses clustered in the same subgroup, the oldest one selected was taken as reference. The 11 prototype viruses were HPAIV A/poultry/Italy/330/97 (H5N2), HPAIV A/chicken/Italy/5093/99 (H7N1), LPAIV A/chicken/Nanchang/7-010/00 (H3N6), LPAIV A/mallard/Netherlands/12/00 (H7N3), LPAIV A/duck/Mongolia/54/01 (H5N2), LPAIV A/mallard/Italy/37/02 (H5N3), HPAIV A/chicken/Netherlands/1/03 (H7N7), LPAIV A/duck/Denmark/65047/04 (H5N2), LPAIV A/duck/Italy/775/04 (H5N3), LPAIV A/turkey/1258/05 (H5N2) and LPAIV A/teal/Italy/3931-38/05 (H5N2). The colour of a viral segment (Fig. 2) corresponds to that of the original virus lineage. **Molecular characterization.** The deduced amino acid sequences of each gene were obtained using MEGA3.1 software. In addition, the lengths of the French AIV PB1 and PB1-F2 proteins were compared with those of the proteins currently available in the influenza virus database (NCBI Influenza Resource).

**ACKNOWLEDGEMENTS**

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**REFERENCES**


