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Research paper

Emergence and multiple reassortments of French 2015–2016 highly pathogenic H5 avian influenza viruses

Briand F.X.[a,*], Niqueux E.[a], Schmitz A.[a], Hirchaud E.[a], Quenault H.[a], Allée C.[a], Le Prioux A.[a], Guillou-Cloarec C.[a], Ogor K.[a], Le Bras M.O.[a], Gares H.[b], Daniel P.[c], Fediaevsky A.[d], Martenot C.[a], Massin P.[a], Le Bouquin S.[a], Blanchard Y.[a], Eterradossi N.[a]

[a] Anses, Université Bretagne-Loire, Ploufragan, France
[b] Laboratoire Départemental d'Analyses et de Recherche, Coulounieix Chamiers, France
[c] Laboratoire des Pyrénées et des Landes, Mont-de-Marsan, France
[d] Ministère de l'Agriculture, Paris, France

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ABSTRACT

From November 2015 to August 2016, 81 outbreaks of highly pathogenic (HP) H5 avian influenza virus were detected in poultry farms from South-Western France. These viruses were mainly detected in farms raising waterfowl, but also in chicken or guinea fowl flocks, and did not induce severe signs in waterfowl although they did meet the HP criteria. Three different types of neuraminidases (N1, N2 and N9) were associated with the HP H5 gene. Full genomes sequences of 24 H5HP and 6 LP viruses that circulated in the same period were obtained by next generation sequencing, from direct field samples or after virus isolation in SPF embryonated eggs. Phylogenetic analyses of the eight viral segments confirmed that they were all related to the avian Eurasian lineage. In addition, analyses of the "Time of the Most Recent Common Ancestor" showed that the common ancestor of the H5HP sequences from South-Western France could date back to early 2014 (± 1 year). This predated the first detection of H5 HP in poultry farms and was consistent with a silent circulation of these viruses for several months. Finally, the phylogenetic study of the different segments showed that several phylogenetic groups could be established. Twelve genotypes of H5HP were detected implying that at least eleven reassortment events did occur after the H5HP cleavage site emerged. This indicates that a large number of co-infections with both highly pathogenic H5 and other avian influenza viruses must have occurred, a finding that lends further support to prolonged silent circulation.

1. Introduction

Influenza A viruses, belonging to the *Alphainfluenzavirus* genus within family *Orthomyxoviridae*, are enveloped viruses (ICTV, 2017). Their negative-stranded RNA genome consists of 8 segments encoding 10 to 14 proteins. 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 in birds and may occur in any combinations (Olsen et al., 2006). AIV genetic evolution is driven by mutations (substitutions, insertions, deletions) and reassortments (Webster et al., 1992). In HAs belonging to the H5 or H7 subtypes, mutations during viral replication, such as substitutions or insertions due to polymerase stuttering, may result in the accumulation of nucleotides encoding several basic amino acids at the cleavage site that is essential to the proteolytic processing of the HA0 protein precursor into its HA1 and HA2 subunits. The resulting polybasic cleavage site allows the so-called highly pathogenic (HP) viruses to replicate efficiently in all organs and to cause a severe and often fatal systemic disease, as opposed to the non-polybasic cleavage site of the low pathogenic (LP) AIVs, whose replication is restricted to the respiratory or digestive tracts, with moderate clinical consequences unless the disease is aggravated by secondary pathogen infections (Pantin-Jackwood and Swayne, 2009). Cleavage site mutations leading to increased pathogenicity were reported in Italian H5N2 in 1997, Italian H7N1 in 1999, Dutch H7N7 in 2003, panzootic "Gs/Gd/96-like" H5Nx and more recently Chinese H7N9 (Capua and Marangon, 2000; Capua et al., 1999; Elbers et al., 2004; Guan et al., 2002; Lee et al., 2017; Pulit-Penaloza et al., 2015; Qi et al., 2017). Because of this ability of H5 and H7 AIVs to evolve from LP to HP with a possible devastating

* Corresponding author at: Anses, Laboratoire de Ploufragan/Plouzané, Laboratoire National de Référence pour l'influenza aviaire, Unité de Virologie Immunologie, Parasitologie Aviaire et Cunicole, Université Bretagne-Loire, B.P 53, Ploufragan 22440, France.
E-mail address: francois-xavier.briand@anses.fr (F.X. Briand).

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impact on the poultry industry, the presence of H5 or H7 LPAIV in poultry is notifiable and regulated at the national and international levels (European Commission, 2005). A further major evolutionary mechanism of AIVs is their capacity to undergo reassortment, whereby co-infection of the same host cell by two or more AIV strains results in progeny virions carrying a mixed set of genome segments derived from both parent viruses resulting consequently in a possibly new phenotype. This phenomenon has given rise to pandemic (Chen, 2009; Kawaoka et al., 1989) or panzootic viruses, and has been extensively demonstrated in recent years among the HP H5Nx AIVs belonging to the 2.3.4.4 clade that has spread worldwide (Lee et al., 2017).

On the 24th November 2015, a highly pathogenic (HP) H5N1 AIV was detected in South-Western France. Its genetic analysis revealed a HA gene belonging to the Eurasian phylogenetic lineage but different from the Gs/Gd/1/96-like zoonotic lineage, with a previously unreported polybasic cleavage site and no zoonotic potential. The related field-investigations quickly allowed the detection of three genetically-related H5HP AIVs exhibiting different neuraminidases (N1, N2, N9) (Briand et al., 2017b). Although several farms with terrestrial poultry experienced clinical disease, the H5HP viruses were mostly detected in domestic duck farms, especially at the free-range and subsequent foie-gras production stages. Clinical signs were occasionally observed in these ducks, however most cases (76%) were asymptomatic infections detected through systematic sampling implemented for virological surveillance in duck flocks (Scoizec et al., 2017).

Indeed, to fight against the spread of H5 HP viruses, a combination of depopulation, zoning and pre-movement RT-PCR analysis was implemented by French authorities in a large restriction zone (RZ) encompassing 17 departments in South Western France, and nearly 65,000 poultry production units. From the 18th January to the 16th May 2016, all duck farms within the RZ were progressively depopulated, cleansed, disinfected, and submitted to a 45-day-long fallowing period. Clinical and virological surveillance were continued during progressive ZR depopulation. Restocking was allowed after improved training, improved biosecurity measures and sanitary control visits were implemented (Fig. S1). Altogether, between the 24th November 2015 and 28th April 2016, this unprecedented surveillance program for Avian influenza allowed to detect 77 cases of infection by H5 HP AIVs, and 20 cases of infection by H5 LP AIVs.

In the present paper, the full genomes of 21 additional H5 HP, 3 new H5 LP and 3 other AIVs isolates collected in South-Western France during the 2015–2016 epizootic were sequenced and compared to the three genomes previously reported (Briand et al., 2017b). These analyses were used i) to better understand how and when the French 2015–2016 H5 HP did emerge and ii) to estimate the extent of reassortment events that occurred since they emerged.

2. Materials and methods

2.1. Sample selection

Cloacal and oropharyngeal swabs were collected for official diagnosis and placed in 2 mL of minimal essential medium supplemented and transferred to local screening laboratories. Screening for H5 viruses, followed by pathotyping and subtype determination by National Reference Laboratory (Anses-Ploufragan, France) were performed as described previously (Briand et al., 2017b). This process routinely includes partial sequencing of the HA and NA genes, using conserved primers that allow co-amplification of the different HA and NA subtypes, should co-infection occur in the sample (Briand et al., 2011). The studied viruses were selected based i) on viral genome quantity (estimated by the Ct value of the detection rRT-PCR M gene), ii) on their genetic diversity as detected from partial HA and NA sequencing and iii) based on their geographic and temporal distributions, so as to maximize the diversity in the studied virus panel. The characteristics of the samples were described in the supplementary Table S1.

2.2. Library preparation, full genome sequencing and NGS data analysis

Viral RNA was extracted with RNeasy® mini kit, Qiagen either from allantoic fluid after egg isolation, or from the original swab sample when isolation in eggs was negative (virus load of the original sample – as determined by rRT-PCR screening – permitting). Libraries, NGS sequencing and analyses were performed as described in (Briand et al., 2017b). For each virus, a consensus sequence was determined.

2.3. Accession number of nucleotide sequences

All sequences are available from the GISAID database (accession numbers Table S1) https://www.gisaid.org/.

2.4. Phylogenetic analyses, molecular clock phylogeny and clustering

2.4.1. Phylogenetic analyses

To perform the phylogenetic analyses, each viral sequence was compared to AIV sequences from the GISAID database (July 2017) and the closest sequences were included in the phylogenetic analyses. Sequences of each segment were aligned using the ClustalW aligner (Thompson et al., 1994) provided in MEGA version 7 (Kumar et al., 1994). Phylogenetic trees were inferred under Neighbor-joining (NJ) and maximum-likelihood (ML) criteria with the Kimura-2-parameter and the GTR + G + I nucleotide substitution model, respectively. Tree robustness was determined through bootstrap analysis of 1000 sequence pseudo-replicates. The H5 HP sequences were also compared with the sequences from non H5HP French viruses to tentatively identify any potential gene donor to French 2015–2016 H5 HP viruses.

2.4.2. Molecular clock phylogeny

Bayesian coalescent phylogenetic analyses were implemented with the BEAST software package (Drummond et al., 2012) to estimate the Time of the Most Recent Common Ancestor (tMRCA) of the studied HP H5 virus sequences. The uncorrelated log-normal relaxed molecular clock with the SDR06 model of nucleotide substitution was applied with constant size demographic models (Lee et al., 2014; Shapiro et al., 2006). For each gene, the datasets were composed by French sequences and 55 to 109 Eurasian sequences. These datasets were run between 30 and 100 million generations depending on the segment, with sampling evolutionary parameters every 3000 to 10,000 generations. The trace files were visualized with Tracer 1.6, especially to check that the effective sample size values were > 200, which corresponds to an acceptable number of independent samples (Shapiro et al., 2006). Maximum clade credibility (MCC) trees were generated after removing a 10% burn-in with TreeAnnotator v1.7.5. The resulting trees were visualized and annotated with the Figtree v1.4 software.

To assess the minimum number of reassortment events that took place since the estimated date of appearance for the French H5HP viruses, the results from tMRCA analyses were compared between the H5 HA gene, and the other genes of H5 HP viruses. Only phylogenetic clusters that i) included French sequences, ii) were supported by bootstrap and posterior values > 75 and > 0.9, respectively, and iii) had a tMRCA posterior to the estimated date of appearance of the cleavage site of H5 HP (including the 95% highest posterior probability), were taken into account in the analyses. Different H5HP isolates were considered to belong to the same genotype when their eight genome segments belonged to the same phylogenetic groups, as defined above. Conversely, H5 viruses with genome segments belonging to different genetic clusters were considered to have emerged by reassortment (Fig. 4.).

Bayesian skyline plots (BSP) were used to investigate the demographic history of French H5HP. This analysis was performed on the 24 French H5HP gene and the 6 H5 gene sequences with the closest