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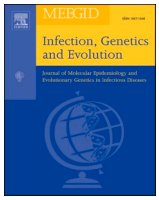

Short communication

Concomitant NA and NS deletion on avian Influenza H3N1 virus associated with hen mortality in France in 2019

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ABSTRACT

An H3N1 avian influenza virus was detected in a laying hens farm in May 2019 which had experienced 25% mortality in Northern France. The complete sequencing of this virus showed that all segment sequences belonged to the Eurasian lineage and were phylogenetically very close to many of the Belgian H3N1 viruses detected in 2019. The French virus presented two genetic particularities with NA and NS deletions that could be related to virus adaptation from wild to domestic birds and could increase virulence, respectively. Molecular data of H3N1 viruses suggest that these two deletions occurred at two different times.

Avian influenza is a contagious disease that can affect all bird species. Wild birds are the natural reservoir of the low pathogenic avian influenza virus (LPAIV) and may transmit these viruses to poultry (Stallknecht and Shane, 1988). AIVs are classified into subtypes based on antigenic differences in their surface glycoproteins (haemagglutinin, HA; and neuraminidase, NA). Sixteen haemagglutinin (H1–H16) and nine neuraminidase (N1–N9) subtypes have been reported in birds (Olsen et al., 2006). They are also classified according to their pathogenicity induced in chickens into high pathogenic avian influenza (HPAI) viruses and low pathogenic avian influenza (LPAI) viruses (European Food Safety et al., 2019). All non-H5/H7 AIV subtypes reported so far are LPAIVs, with very few exceptions (Liu et al., 2003; Wood et al., 1996), restricted to the respiratory or digestive tracts, with mild to moderate clinical consequences unless the disease is worsened by secondary pathogen infections (Lee et al., 2020; Pantin-Jackwood and Swayne, 2009).

In January 2019, a first case of H3N1 avian influenza (AI) was detected in Belgium in laying hens. Eighty-two other H3N1 outbreaks were thereafter identified in the North-West of Belgium with an increased number of cases in May and June (Steensels et al., 2020). Clinical signs mainly affecting layer or breeder hens were observed, triggering up to 60% mortality. In addition, in laying hens the complete capacity of lay was lost, and not regained thereafter making the flocks an economic loss. In May 2019, in Northern France, close to the Belgian border, symptoms suggestive of avian influenza infection were observed in a farm of 8200 breeding hens. Drop in feed consumption and egg production, together with egg shell fragility were observed. During the first three days after the onset of clinical signs, the mortality rate was 1%, 2% and 6% and cumulated mortality reached 25% after 10 days (ESA, 2019). The flock was finally culled 10 days after the first symptoms. Two other H3N1 cases were also detected in France, one from environmental sample and the last one exhibited similar deletion feature as Belgian sequences. In this study, we investigate phylogenetic and molecular features of this first detected H3N1 virus that could explain the virulence observed in chickens.

Oropharyngeal and cloacal swabs, as well as organs (brain, lung, and intestines) were collected from affected animals. Individual swab and organ supernatants were pooled by five for analyses. Viral RNA extracted was tested using M-gene, H7-gene and H5 gene real-time reverse transcription-PCRs (rRT-PCRs) by the district laboratories

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Table 1
Percent nucleotide identies of the eight complete coding sequences between the French Influenza H3N1 virus sequences and their closest sequences.

Segment	Closest Belgian strain	Accession number	Nucleotide identities	No Belgian closest strain	Accession Number	Nucleotide identities
PB2	A/*Gallus gallus*/Belgium/3497_0001/2019(H3N1)	MN006987.1	99.91	A/tufted duck/Georgia/1/2012(H2N3)	MF147767.1	96.65
PB1	A/*Gallus gallus*/Belgium/3497_0001/2019(H3N1)	MN006986.1	99.91	A/northern shoveler/Egypt/MB-D-695C/2016(H7N3)	MN208053.1	98.52
PA	A/*Gallus gallus*/Belgium/3497_0001/2019(H3N1)	MN006985.1	99.86	A/black-headed gull/Netherlands/21/2014(H11N1)	MF575095.1	98.66
HA	A/Turkey/Belgium/4539/2019 (H3N1)	MN826752.1	99.88	A/Mallard/Netherlands/37/2015 (H3N8)	MK414733.1	98.81
NP	A/*Gallus gallus*/Belgium/3497_0001/2019(H3N1	MN006983.1	100	A/duck/Mongolia/543/2015(H4N6)	LC121413.1	98.45
NA	A/Turkey/Belgium/4539/2019 (H3N1)	MK972682.1	99.85	A/mallard duck/Georgia/7/2015(H6N1)	MF694086.1	97.82
M	A/*Gallus gallus*/Belgium/3497_0001/2019(H3N1)	MN006981.1	99.9	A/mallard/Netherlands/89/2017 (H4N6)	MK192396.1	98.81
NS	A/Turkey/Belgium/4539/2019 (H3N1)	MN826775.1	99.85	A/mallard duck/Netherlands/31/2013 (H10N7)	KX979173.1	95.83

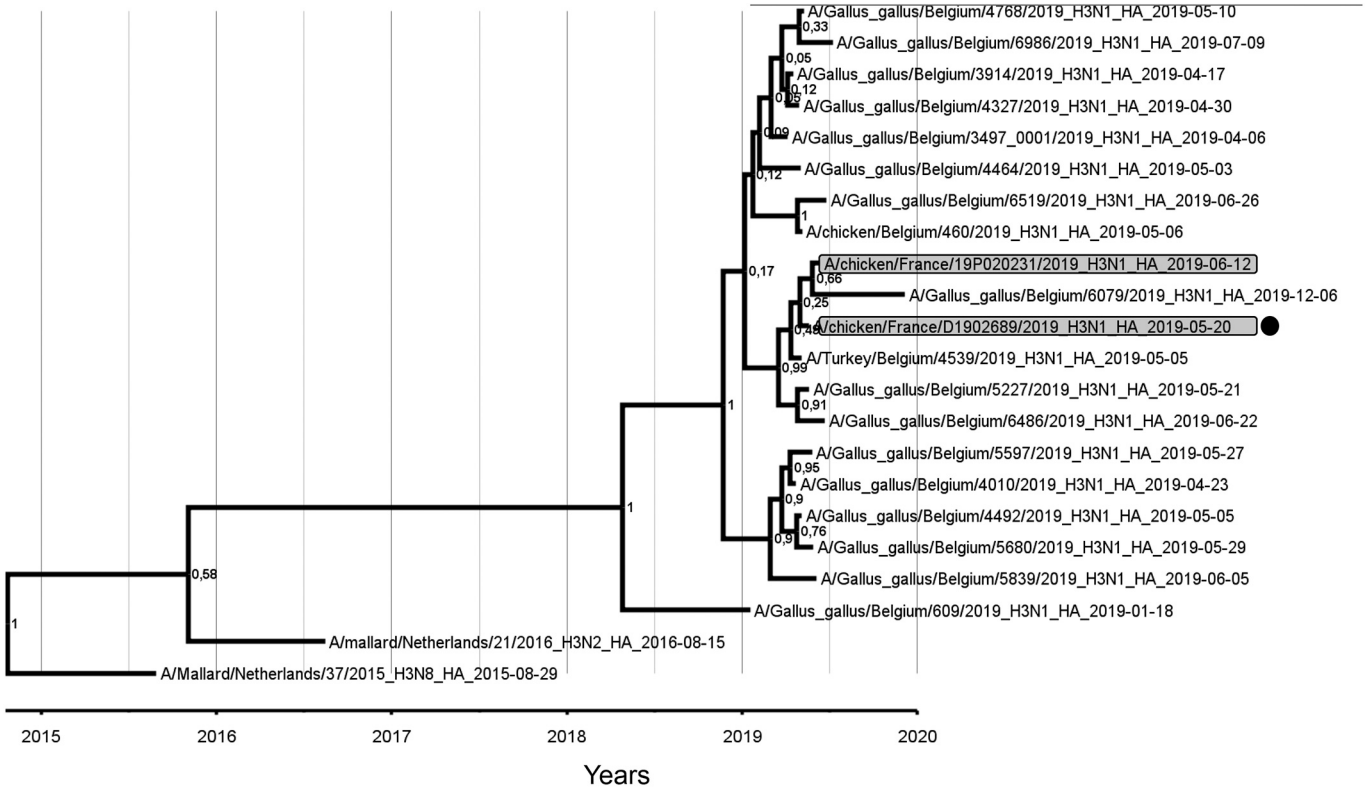

Fig. 1. Maximum credibility tree of the hemagglutinin H3 genes and their closest related sequences. Trees were generated using BEAST software 1.8 using SDR06 substitution model and an uncorrelated relaxed clock model. Posterior probabilities are indicated for each node. French H3N1 sequences are highlighted in grey. Sample with double NA and NS deletion was indicated with a black dot.

(Slomka et al., 2007). All samples were positive with M-gene real-time RT-PCR and negative for the H5-gene and H7-gene. Virus isolation from organs was performed according the manual of diagnostic (Anonymous, 2009). This isolated virus was called A/chicken/France/D1902689/2019. Viral RNA extracted from allantoic fluids and directly from pools of supernatant samples were amplified by RT-PCR (Zhou et al., 2009) and the full genomes were sequenced using the Ion Torrent technology (Life Technologies). The viral sequences were obtained by *de novo* assembly of the reads with Spades. Each segment was aligned with the closest available sequences from GISAID and Genbank using ClustalW. Phylogenetic analyses were performed with Neighbor-joining method and Tamura-3-parameter model using MEGA7 (1000 bootstrap replicates). Bayesian coalescent phylogenetic analyses were implemented with the BEAST software package (Drummond and Rambaut, 2007) to estimate the Time of the Most Recent Common Ancestor (tMRCA). The uncorrelated log-normal relaxed molecular clock with the SDR06 model of nucleotide substitution was used with constant size demographic models. The number of MCMC step was run with 40 million generations to obtain effective sample size values >200.

The analyses revealed the presence of the H3N1 virus in all sequenced samples. In addition, no presence of any other know poultry bacterial and viral poultry pathogens for which may explain the severity of the observed symptoms was identified by RNA/DNA sequencing (Next-generation Sequencing) (data not shown) on lungs, intestines and brains. In all samples (isolated virus, and pool of swabs, intestine, brain) exhibited similar viral genome sequences with <6 different nucleotides

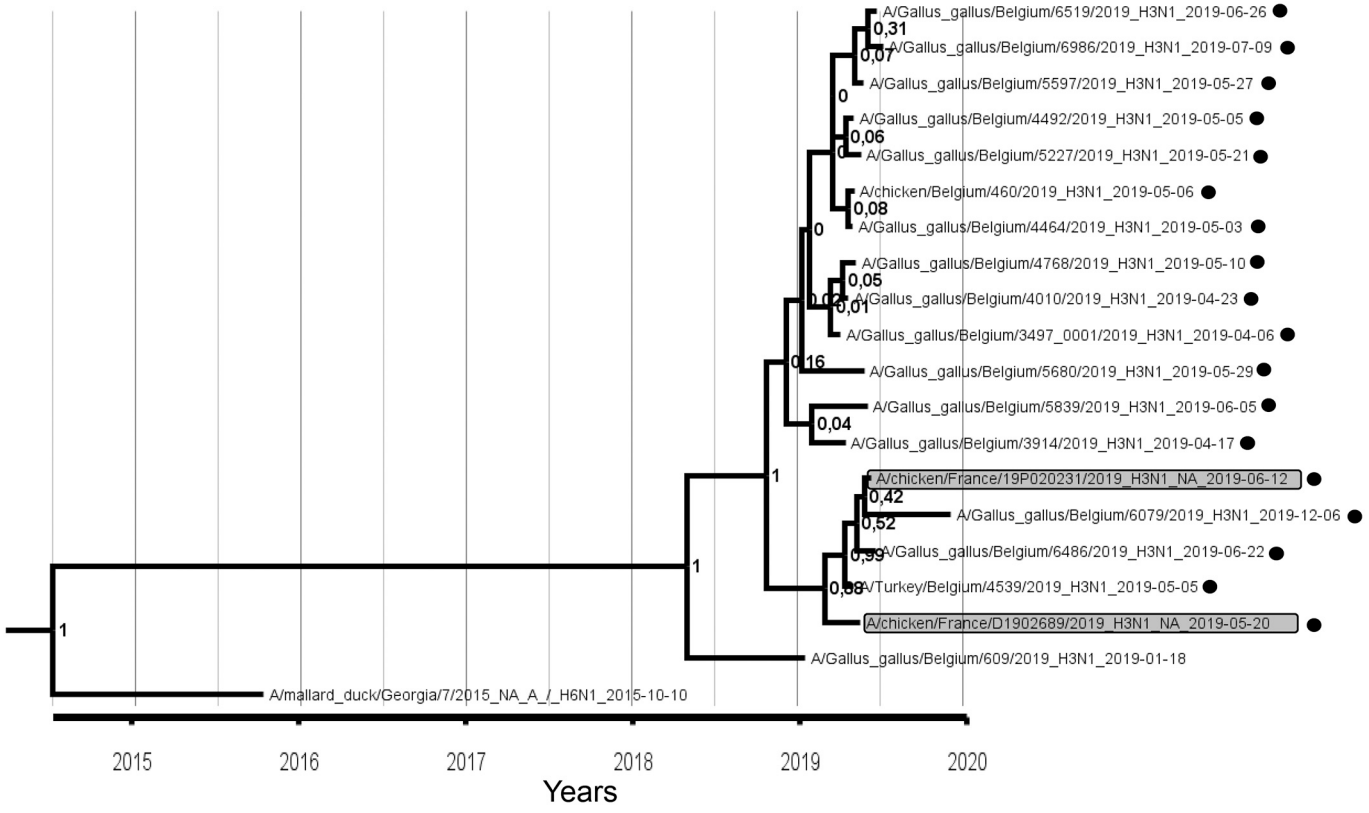

Fig. 2. Maximum credibility tree of the Neuraminidase N1 genes and their closest related sequences. Trees were generated using BEAST software 1.8 using SDR06 substitution model and an uncorrelated relaxed clock model. Posterior probabilities are indicated for each node. French H3N1 sequence are highlighted in grey. Sequences exhibiting the NA deletion were indicated with a black point.

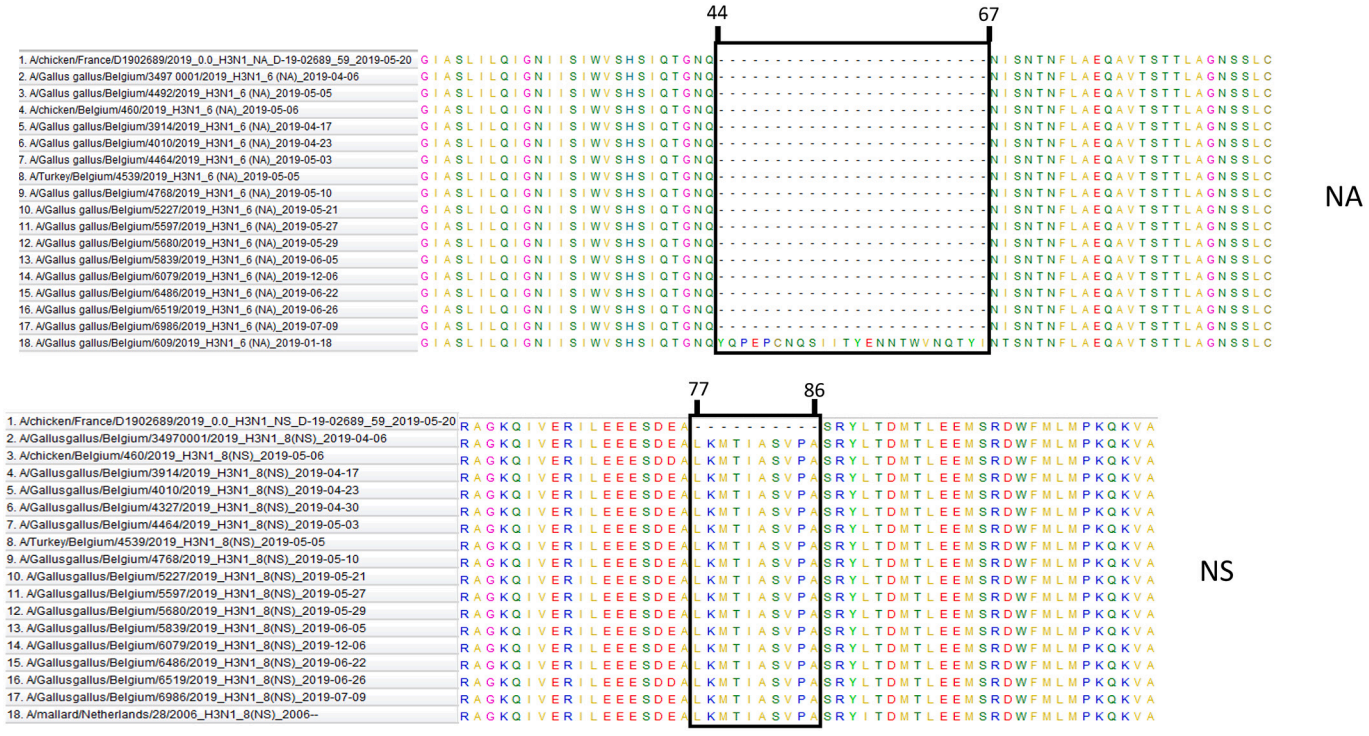

Fig. 3. Partial amino acid alignment of NA and NS sequences of French and Belgian sequences. Black squares correspond to the deletion zones observed in the French NA and NS sequences. Amino acid positions of the deletions are indicated above the alignment. Accession Numbers of Belgian sequences for NA and NS: MK972680, MK972682, MK972684, MN006982, MN435598, MN826762 to MN826774 and MN006980, MN435600 MN826775 to MN826788.

on 13,090 nucleotides between samples. Only the sequences from the isolated virus was used to the phylogenetic analyses that were submitted to GISAID (EPI1701820 to EPI1701827). The eight AIV segments belonged to the avian Eurasian lineage and exhibited 99.85% to 100% nucleotide identities with sequences of Belgian H3N1 viruses detected in the same period (Table 1).

Both H3N1 Belgian and French sequences were closely related to sequences from different continents as from Europe for PB2/PA/HA/M/NS segments, from Asia for NP segment and from Africa for PB1 segment (Table 1). Time resolved phylogenetic tree of the HA gene indicated that the common ancestor of French and all Belgian sequences was dated nearly mid of May 2018 (95% High Posterior Density (HPD): Nov 2016 to Jan 2019) (Fig. 1). For NA, the estimation of the date of the common ancestor of the French and all Belgian sequences was closed to the end of April 2018 (95% HPD, Apr 2017 to Nov 2018) (Fig. 2).

The most atypical features observed on these sequences was the stalk deletion of 24 amino acids observed on the NA protein (positions 44 to 67; Fig. 3) already identified for 17 of the 18 available Belgian complete NA sequences (excepted the first case). The NA deletion was larger than other stalk deletions frequently observed in LPAIVs and involved in adaptation from wild to domestic birds, or in increased virulence in birds (Munier et al., 2010; Stech et al., 2015) and mice (Park et al., 2017). The estimated date of the appearance of deletion in NA was occurred around mid of October 2018 (95% HPD: Mar 2018 to Feb 2019). Additionally, a unique deletion of 10 amino acids on the NS1 protein (positions 77 to 86; Fig. 3) was identified, but not previously reported in the 16 publicly available Belgian sequences. Deletion on NS1 has already been associated with an increase replication potential and pathogenicity of avian H1N1 and HP H5N1 viruses (Long et al., 2008; Trapp et al., 2014). The MCC tree did not given enough good posterior probabilities to obtain a confident estimation date of NS deletion. However, according the topology of the NS tree, which was similar to HA and NA, the date of common ancestor between French sequence (with NS deletion) and the closest relative Belgian sequences (without NS deletion) was to Mar 2019 (HPD%95 5 nov 2018 to 7 may 2019). So by extrapolation, the NS deletion observed in French sequence should be estimated from Mar 2019 to the date of detection of the French H3N1 (20-05-2019).

According to these data, it was likely that the H3N1 viruses found in Belgium and in France, came from a Belgian poultry farm, which evolved with NA 24 amino acid stalk deletion. The virus with deletion of NA spread through the poultry farms in Belgium, and either before the introduction in France or once in France, the virus underwent a deletion on the NS segment. In addition to this H3N1 outbreak, a second case was only detected by a real-time RT-PCR specific to H3 gene on environmental samples. Subsequently a third case of H3N1 was detected in June 2019 from cloacal and oropharyngeal hens samples and birds were immediately culled. The H3N1 segment sequences of the third case (A/chicken/France/19P020231/2019) were closed relative sequences to French and Belgian sequences, but did not exhibited the NS deletion observed in a first H3N1 first outbreak.

This H3N1 virus did not exhibit the major virulence determinant corresponding to polybasic HA cleavage site as observed for high pathogenicity (HP) H5 and H7 avian influenza, which is consistent with intravenous pathogenicity index (IVPI) of 0 performed in specific-*pathogen*-free chickens (Anonymous, 2009)(data not shown). This IVPI value agreed to already obtained results on Belgium strains (Steensels et al., 2020). Nevertheless, clinical symptoms observed in poultry in Belgium and France should be explained by different factors. Firstly, coinfection of low pathogenic AIV and another pathogen can amplified expected clinical symptoms with individual pathogen as already described in a coinfection between *Mycoplasma gallisepticum* and H3N8 virus (Stipkovits et al., 2012). Even if, no other poultry pathogen were detected in French samples, *Mycoplasma gallisepticum* was previously detected in a farm hit by the H3N1 virus. On the other hand, bacterial preinfection could be of benefit to AIV pathogenesis as in cases of bacterial protease that facilitate cleavage of the HA of LPAIV (Samy and Naguib, 2018). Secondly, on Belgian H3N1 viruses, a study have demonstrated that the NA-mutation N130S was implicated in the cleavage of HA allowing the systemic replication of the virus as HPAI viruses (Schon et al., 2021). French H3N1 also exhibited this mutation deleting an N-glycosylation site and allowing the trypsin-independent cleavage of HA.

In addition to these data, the concomitant acquisition of the two NA and NS deletions could be involved in the observed mortality in poultry infected by this H3N1 AIV. The spread of this emerging H3N1 virus associated sometimes with high chicken mortality should be monitored in order to limit the economic losses for the industry and any potential reassortments of the NA and NS segments with the other viruses such as HP H5 or HP H7 viruses that could circulate in Europe (Breed et al., 2010).

Conflict of interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

François-Xavier Briand: Conceptualization, Methodology, Writing – original draft. **Audrey Schmitz:** Writing – review & editing. **Axelle Scoizec:** Writing – review & editing. **Chantal Allée:** Investigation. **Rachel Busson:** Investigation. **Carole Guillemoto:** Investigation. **Hélène Quenault:** Investigation. **Pierrick Lucas:** Data curation. **Isabelle Pierre:** Investigation. **Katell Louboutin:** Investigation. **Cécile Guillou-Cloarec:** Investigation. **Claire Martenot:** Writing – review & editing. **Martine Cherbonnel-Pansart:** Writing – review & editing. **Rodolphe Thomas:** Investigation. **Pascale Massin:** Writing – review & editing. **Florent Souchaud:** Investigation. **Yannick Blanchard:** Writing – review & editing, Data curation. **Mieke Steensels:** Writing – review & editing. **Benedicte Lambrecht:** Writing – review & editing. **Nicolas Eterradossi:** Writing – review & editing, Supervision. **Sophie Le Bouquin:** Writing – review & editing. **Eric Niqueux:** Writing – review & editing. **Béatrice Grasland:** Writing – review & editing, Project administration.

Data availability

The sequences obtained for the current study are deposited in GISAID, under accession numbers EPI1701820 to EPI1701827. All other relevant data are included in the manuscript and the references.

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