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Article

Phylogenetic Analyses of HA and NA Genes of Algerian H5N1 and H5N8 Strains Reveal Putative NA Recombination Hotspots

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Abstract: Highly pathogenic Avian influenza H5Nx became an uncontrolled threat, causing iterative outbreaks in avian and jumping the species barriers to other vertebrates. Two AIV outbreaks caused by H5N8 in 2020-2021 (mortality of 70%) and by H5N1 in 2022-2023 (mortality of 40%) targeted the Algerian poultry flocks from December 2020 to July 2023. Hemagglutinin (HA) and neuraminidase (NA) coding sequences of isolated strains were genetically analyzed by RT-PCR, sequencing, and phylogeny to determine their pathotypes and origins. Analyses of sequencing data confirmed the highest homology of the strains to the described H5N8 and H5N1 strains, respectively, and the presence of a multi-basic cleavage site in the HA aa-sequences confirmed the highly pathogenic property of both subtypes. Closer phylogenetic analysis of all sequences showed genetic proximity with African, European, and Asian strains. While all genes seem to be of Eurasian lineage, the N1 gene consensus was closer to both. Recombination events and stalk deletion were revealed in N1 genes. These findings have encouraged us to explore the two proteins for a better understanding of their antigenic interactions.

Keywords: Phylogenetic analyses; H5N1; H5N8

Introduction

Highly pathogenic avian influenza (HPAI) viruses are members of the Alpha-influenza viruses (AIVs) that are part of the Orthomyxoviridea family (1). AIVs are classified into several subtypes according to their antigenic determinants in the hemagglutinin (HA) and neuraminidase (NA) proteins. There are 18 distinct HAs and 11 distinct NAs identified till now (2). AIVs have a segmented RNA genome and an RNA-polymerase that lacks the 3'-5' exonuclease activity, which results in an accumulation of an average mutation rate of 10^{-4}. The variations observed in the genome, which induce aa changes, are known as (i) antigenic drift, that represent the accumulation of mutations leading to considerable changes in both HA and NA peptides, and (ii) antigenic shift, that leads to a virion with a hybrid genome composed of different segments exchanged from various strains during the co-infection of the same cell (1,3-6). On the other hand, recombination is not a process that is well studied or proven among influenza viruses, even though evidence of homologous recombination has been demonstrated on different segments in Chinese isolates (7,8).

AIVs are divided into two pathotypes that are either low (all subtypes) or high pathogenic (H5 and H7), as the latter not only target the respiratory and digestive systems, like the low pathogenic strains, but also cause systemic degenerative disease in infected avian hosts (9). This high

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pathogenicity was shown to be associated with the presence of specific multi-basic amino acids within the cleavage site on the HA protein targeted by subtilisin-like enzymes. The cleavage activity of these enzymes, present in various organs, is responsible for the differentiation of HA into its active form (10, 11). Migratory birds, particularly *Anseriformes* and *Charadriiformes*, are considered the natural reservoir of AIVs (12). They are known to host the low pathogenic pathotype; however, when the virus is transmitted to poultry flocks, there is a change toward the high pathogenic form (13). The change is mostly indels or single-nucleotide deletions (SNDs) in the HA RNA that are required for better adaptation to the host (14)

Globally, H5Nx strains are associated with two different lineages, the Eurasian lineage, which originated from China in 1996 (A/goose/Guangdong/1/1996) [Gs/GD], and the North American lineage that was identified later in 2014 (13,15-18). In Africa, several migratory routes that could be associated with virus transmission to the continent were identified. Moreover, three different clades of the Gs/GD lineage {2.2 (2005–2011), 2.3.2.1c (2011–2017), and 2.3.4.4b (2014–2018)} that originated from East or North-Central Asia were identified (19,20). The HPAI H5N1 2.3.4.4b is the most concerning group as it became a threat not only to the farm poultry production but also to other species, since spillovers to dairy cattle across several states in the USA and four farm workers were recently reported (20-23). This cluster is widely distributed in Asia and Europe, causing tremendous losses in poultry production, and it was introduced in December 2021 into North America, and ever since, humans, avian, and mammal species have been found affected (24-27). During the last four years, at least 35 documented cases of AIV/H5N1 virus infections in humans have been reported to WHO (24). The majority of these patients were infected with viruses belonging to the clade 2.3.4.4b (24,28,29). A total of 66 cases of HPAI A(H5N1) belonging to the clade 2.3.4.4 have been reported in infected humans in the US in 2024 following contact with infected poultry or cows (24,29).

Two distinct epizooties have affected the poultry flocks raised in Algeria; the first one, from December 2020 to May 2021, was caused by H5N8, and the second, from late September 2022 until July 2023, was caused by H5N1. They have caused up to 70% and 40% mortality in chickens and turkeys, respectively. Previously, we reported on the detection and the pathological properties of viruses that caused both epizooties (30). In this study, we are reporting on the genetic characteristics of the HA and NA genes of these strains following sequence analyses.

Materials and Methods

RNA extraction was performed using Qiagen products on organ samples collected during both epizooties as described in Ammali et al., 2024. RT-PCR was performed using the primers H5N1-ha_4Fv2 and H5N1-ha_4Rv2 to amplify the full *hemagglutinin* (*HA-H5*) gene and the primers H5N1-na_6Fv2 and H5N1-na_6Rv2 to amplify the full *neuraminidase* (*NA-N1*) gene (31). The full NA-N8 gene was amplified using H5N8-N8-F and H5N8-N8-R primers we reported earlier, Ammali et al. 2024 (30). The same thermal profile was used for amplification of all the genes with one-step reverse transcription at 50°C for 15 min and denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 15 sec, 50°C for 30 sec, and 72°C for 2 min.

Semi-nested PCR protocols were conducted for the samples that failed to produce the full-length HA amplicon products with the external primers. One µl of the PCR products was used as a matrix for a new PCR amplification using the external H5N1-ha_4Fv2 associated with the internal H5-1162Rv: 5′-GAGTGGATTCTTTGTCTG-3′ (31,32) primers, to generate the 1155 bp HA-H5 fragment. Similarly, the external H5N1-ha_4Rv2 was associated with the internal H5-1063Fw: 5′-TTTATAGAGGGAGGATGG-3′ (31, 32) primers to generate the 716 bp HA-H5 fragment. These two fragments cover the total HA-H5 coding sequences.

A similar semi-Nested PCR strategy was performed using the external H5N1-na_6Fv2 and H5N1-na_6Rv2NA-N1 primers (31) on extracted RNAs, and then the internal N1av-648Rv: 5′-GCCATTTACACATGCACATT-ATTCAG-3′ (32) and AN1B: 5′-TTGCTTGGTCAGCA -AGTGCA-3′ (33) NA-N1 primers on the first PCR products. These Semi-nested PCR reactions generated 648 bp and 895 bp fragments, respectively, covering the total NA-N1 coding sequences.

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For the NA-N8 gene, we used the H5N8-N8-F and H5N8-N8-R external primers in combination with NA8-856-R: 5′-GCATTCCACTTTACCATCA-3′ and NA8-735-F: 5′-GTAATGACTG-ACGGTCCAT-3′, respectively. These primers were designed with BLASTN (34) (RRID: SCR_001598) and BioEdit v7.2.5 (RRID: SCR_007361). These semi-nested PCR amplified 835 bp and 665 bp amplicons, respectively.

The thermal profile for the amplification applied to all semi-nested PCR reactions was identical to the one used with the external primers, but without the reverse transcription step. Samples of the resulting amplicon products were separated in a 1.2% agarose gel by electrophoresis containing safe view dye and then visualized under UV light.

PCR products and gel-separated PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (MACHEREY-NAGEL) according to the protocol provided. The quality of purification was assessed by electrophoresis using a 1.2% agarose gel.

The sequencing was conducted on thirteen H5N8 samples from the first epizooty (2020-2021) and eight H5N1 from the second epizooty (2022-2023) isolated in Algeria (30).

Purified DNA samples were referred to Eurofins Genomics Europe (Ebersberg, Germany) using the LightRun service for sequencing based on the Sanger method. The DNA template and the oligonucleotide primer mixes were prepared according to the protocol applied for the LightRun service.

The sequences were treated with SnapGene (RRID:SCR_015052), the alignment was achieved using Clustal Omega version 1.2.4 and phylogenetic analyses were conducted with BLASTN (34), Phylogeny.fr (35) (RRID:SCR_010266), and MEGA 11 Software (RRID:SCR_000667) (36) using the Neighbor-Joining method (37). The evolutionary distances were computed using the Maximum Composite Likelihood method (38). Recombination hotspots were detected with Recombination Detection Program (RRID:SCR_018537) (RDP4, v.4.39) (39) and Genetic Algorithm for Recombination Detection (GARD) (https://www.datamonkey.org/gard) (40).

Results

Sample Amplification and Sequencing

The PCR aiming the full-length genes successfully amplified the HA-H5 coding sequences in nine samples identified as H5N8 and five others identified as H5N1. Similarly, the full-length NA-N8 coding sequences were amplified in nine samples identified as H5N8, and the NA-N1 coding sequences from five samples were identified as H5N1. Following amplicon purifications, eight H5N8 and five H5N1 were positively selected for DNA sequencing.

The semi-nested PCRs applied on the samples that failed to amplify enough amplicon with the external primers, provided additional HA-H5 amplicons in six samples, NA-N8 amplicons in four additional samples, and NA-N1 amplicons in two additional samples. Therefore, four H5N8 and two H5N1 purified PCR products were selected for sequencing.

The sequencing data based on the classical Sanger method using the external primers provided the full-length sequences of two HA-H5 genes (PP422362 (H5N8) and PP422967(H5N1)), and 12 partial sequences of HA-H5 genes (five from H5N1 and eight from H5N8) (Table 1). The full-length sequences of NA-N1 were obtained with two samples (PP422536 and PP422956), and partial sequences in three others (Table 1). For the NA-N8 coding sequences, partial sequences were obtained in ten samples (Table 1). Sequencing annotation and aligning of the HA and NA genes from the two subtypes were conducted separately. The details regarding the sequences are reported in Table 1.

Table 1. Details of the sequenced samples.

	Farm code	Localization/date	Host	Genes	GeneBank accession	Length (bp)
H5N8	E1	Mostaganem (12/2020)	Chicken	H5	PP422233	1680
				N8	PP411896	1379

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	E15	Tissemsilt (02/2021)	Chicken	H5	PP422235	1304
				N8	ND	ND
	E20	Djelfa (04/2021)	Turkey	H5	PP422366	1695
				N8	PP411939	1258
	E27	Djelfa (03/2021)	Chicken	H5	PP422392	1695
				N8	PP422224	1267
	E30	Djelfa (04/2021)	Chicken	H5	PP422393	1612
				N8	PP422225	1053
	E33	Djelfa (04/2021)	Turkey	H5	ND	ND
				N8	PP407304	1221
	E39	Djelfa (04/2021)	Chicken	H5	PP422394	1614
				N8	PP422228	1096
	E40	Bouira (04/2021)	Chicken	H5	ND	ND
				N8	PP422226	1346
	E42	Boumerdes (04/2021)	Chicken	H5	PP422361	1686
				N8	PP422230	1206
	E44	Tiaret (05/2021)	Turkey	H5	PP422365	1498
				N8	PP411909	1247
	E48	Blida (05/2021)	Chicken	H5	PP422362	1714
				N8	PP422219	1335
H5N1	E85	Médéa (09/2022)	Chicken	H5	PP422959	1703
				N1	PP422535	1396
	E114	Sétif (12/2022)	Chicken	H5	PP422967	1729
				N1	PP422536	1458
	E120	Boumerdes (01/2023)	Chicken	H5	PP422968	1695
				N1	PP422958	1135
	E121	Tipaza (01/2023)	Chicken	H5	PP422973	1313
				N1	PP422956	1442
	E150	Médéa (05/2023)	Turkey	H5	PP422972	1417
				N1	ND	ND
	E167	Tiaret (07/2023)	Chicken	H5	PP422971	1517
				N1	PP422537	1343

ND= Not determined.

Molecular Characterization of the H5N8 and H5N1 Isolates

Analyses of sequencing data showed that all the sequences of all HA-H5 samples have a conserved motif ^{337}**PLREKRRKR/GLF**^{348} corresponding to the cleavage site typical of the highly pathogenic pathotype of the strains.

Similarly, examination of NA-N8 and NA-N1 sequencing data revealed that the Oseltamivir susceptibility site on N8 (273H and 293N, N8 numbering) and N1 (275H and 295N, N1 numbering) was conserved in the sequences of our isolates (41,42).

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Phylogenetic Analysis

Phylogenetic analysis was performed separately for all the sequence data of the four genes compared to similar sequences available in the Genbank database, which were used for comparison.

Based on this analysis, the HA-H5 genes from the 2020-2021 epizooty caused by H5N8, were closely related to the Nigerian isolate (Chicken/Nigeria/2021(H5N8)) (nucleotide homologies 99.21%) and the Chinese strain (Cygnus Columbianus/Hubei/2020 (H5N8)) (nucleotide homologies 99.20%) (Figure 1.a). These data consolidated our earlier findings that the virus isolates causing this epizooty were indeed H5N8 subtypes. The sequences of the virus isolates also clustered with a variety of other subtypes, that were isolated mainly from wild birds, including the H5N3 isolated in France in 2021 (nucleotide homologies 98.76%-98.85%), the H5N1 isolated in Egypt, Spain, Japan and USA in 2022 (nucleotide homologies 98.68%-98.06%) and the H5N5 isolated in Dagestan and Astrakhan in 2021 (nucleotide homologies 98.94% and 98.85%, respectively) (Fig.1.a). The NA-N8 genes of this H5N8 virus closely clustered with the sequences of the French isolates (Swan/France/2020(H5N8)) and (Mule duck/France/2020(H5N8)) collected in 2020 (nucleotide homologies 99.12%) (Figure 1.b).

Phylogenetic analysis of the sequences of both H5 and NA genes of the viruses causing the 2022-2023 epizooty confirmed that the virus was an H5N1 subtype. The HA and NA sequences of these virus isolates were found to be closer to those of an African H5N1 isolate that was collected in Mauritania in 2022 (Great_White_Pelican/Mauritania/2022 (H5N1)) with 99.25% nucleotide homologies for HA-H5 and 98.74% for NA-N1 genes (Figure 1.c and d). Sequence similarities of these viruses were also found with those of other strains isolated in 2021 and 2023 outbreaks in Egypt, with similarities reaching 98.6% in HA-H5 and 98.34% in NA-N1 genes. The sequences were likewise related to the French strains isolated in 2021 and 2022, with nucleotide homologies in HA-H5 reaching 98.94% and up to 98.59% in NA-N1 (Figure 1.c and d).

To examine to which lineage the sequences of Algerian strains are the closest, we compared their sequences to those of selected representatives of the two lineages from the Influenza Virus Resource database, and calculated the patristic distances (Table 2). Based on the results of these calculations and the phylogenetic trees, all genes were found to be related to the Eurasian lineage (Figure 2.a, b, c, d). However, the N1 gene shared similarities with both lineages; therefore, a Z test (α=0.05, Z=1.96) was conducted, and no significant difference was observed between the two means.

Table 2. Calculated means of patristic distances between the sequences of studied genes and those of representative North-American and Eurasian lineages.

Subtype	Genes	Eurasian lineage	North-American lineage
H5N8	**H5**	**0.05306**	**0.1162**
	N8	**0.04458**	**0.17688**
H5N1	**H5**	**0.08617**	**0.14505**
	N1	**0.08905**	**0.08356**

H5 consensus sequences were compared to representatives' sequences of the Gs/GD lineage clades and both strains belong to the 2.3.4.4b (Figure 3).

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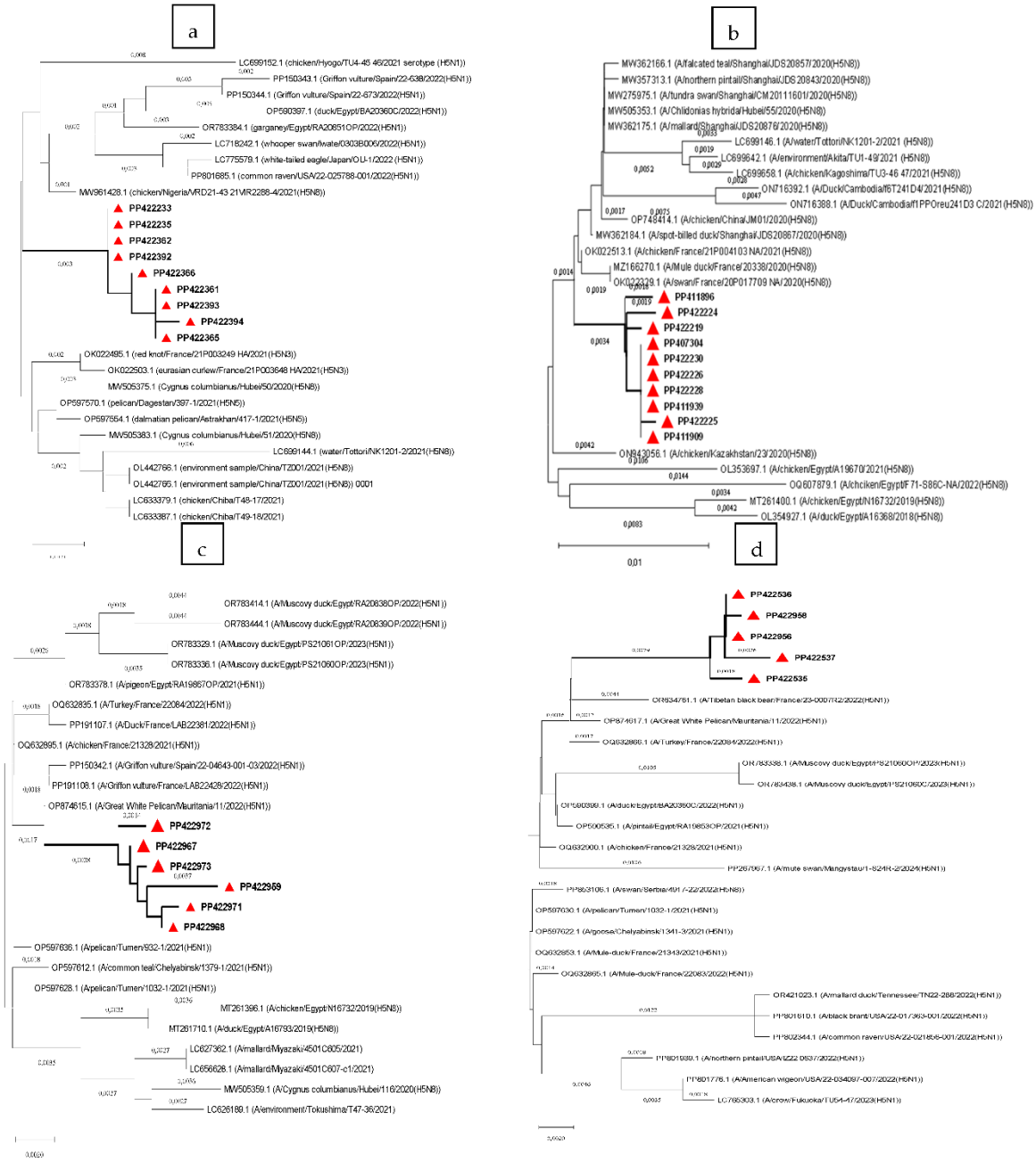

Figure 1. Phylogenetic tree of a) the HA-H5 (H5N8); b) the NA-N8 (H5N8); c) the HA-H5 (H5N1); and d) the NA-N1 (H5N1).

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Figure 2. Phylogenetic analysis of Algerian HA-H5 sequences with those of representative North American and Eurasian sequences. North-American HA-H5 (green branches) and Eurasian HA-H5 (red branches) from (H5N8) lineages are in a). In b), the NA-N8 (H5N8) were similarly plotted In c), the HA-H5 (H5N1) and in d), the NA-N1 (H5N1) are plotted.

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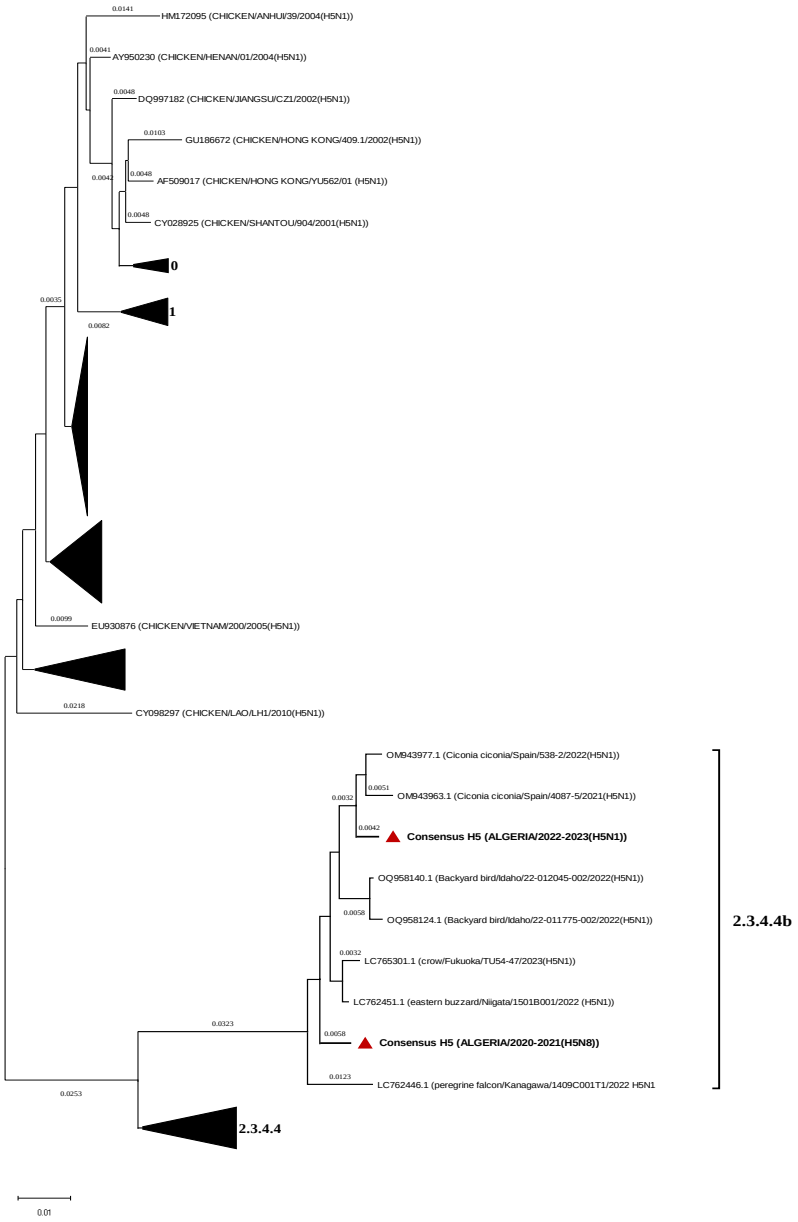

Figure 3. Cladogram of the consensus sequences of Algerian HA-H5 genes compared with those of representative Gs/GD lineage clades.

N1 Gene

Recombination Events Detection

To detect the presence of a putative recombination hotspot in the mismatching region of the gene, we used the RDP 4.39 (39) to test the four N1 sequences. Several methods (RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, PhylPro, LARD and 3Seq) were used to detect the potential hotspot points of recombination. RDP, GENECONV, MaxChi, Chimaera, BootScan and 3Seq methods revealed a recombination hotspot in three (PP422536, PP422956, PP422535) out five analyzed sequences (Table 3). Two potential breakpoints were determined in PP422536 and PP422535, the first one was at position 15 and the second at 1380, and one breakpoint in PP422956 beginning at 1380 and ending at 1456 (according to the reference sequence PP422536 alignment) (Figure 4.a). The analysis established PQ724324.1 (A/duck/Egypt/BA20361C/2022(H5N1)) as the major parent and FJ032003.1 (A/mallard/ON/499/2005 (H5N1)) as the minor parent. The pairwise identity ranged between 49%

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and 99% between the two breakpoints. Regarding the minor parent, pairwise identity was mostly lower than 10% compared to the N1 recombinants tested.

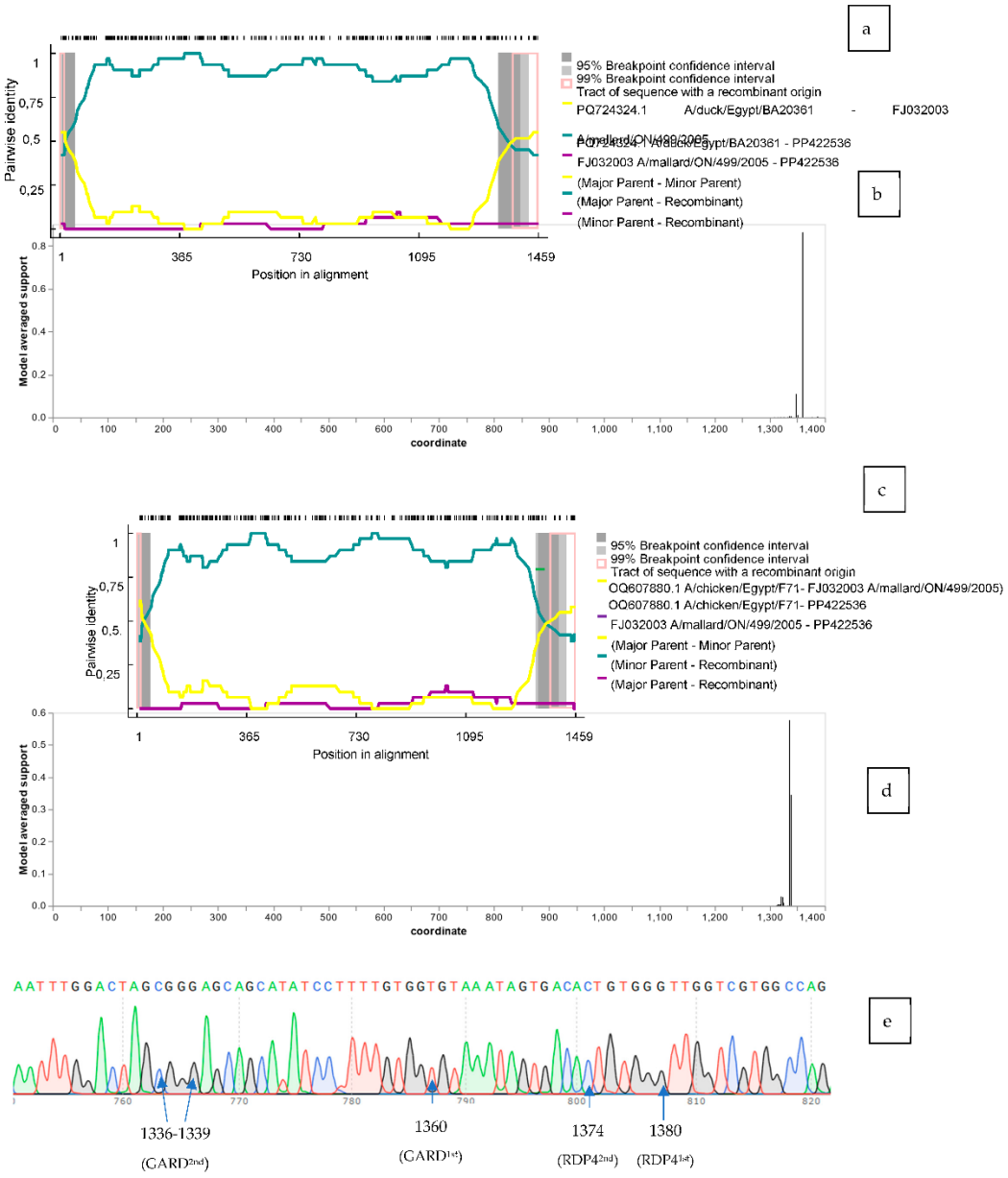

Figure 4. Recombination analysis on the N1 genes. a) RDP 4 results of the PP422536 sequence, PQ724324.1 (A/duck/Egypt/BA20361C/2022(H5N1)) as the major parent in green, FJ032003.1 (A/mallard/ON/499/2005(H5N1)) as the minor parent in purple, identity comparison between the major and the minor parent is shown in yellow. b) GARD (Genetic Algorithm for Recombination Detection) results showing the potential hotspot at the position 1360, c) RDP4 2nd Attempt results of the PP422536 sequence, OQ607880.1 (A/chicken/Egypt/F71-F114C/2022(H5N1)) as the major parent in green, FJ032003.1 (A/mallard/ON/499/2005(H5N1)) as the minor parent in purple, identity comparison between the major and the minor parent is shown in yellow, d) GARD 2nd Attempt results showing the two potential hotspots at positions 13,336 and 1339, e) Chromatogram of the region carrying potential recombination hotspots (PP422536: 1324-1394).

Similar analysis was conducted with Genetic Algorithm for Recombination Detection (GARD) available in the Datamonkey web server (40). Similar results were found where a potential breakpoint

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was identified at position 1360 (86%) (according to the reference sequence PP422536 alignment) (AIC-$c_{baseline}$ = 7844.9, c-$AIC_{breakpoint}$ = 7729.2) (Figure 4.b).

The latter analysis failed to determine the minor parent as the closest to the C terminal last 100 bp of our sequence, therefore that part was blasted on NCBI and the best three hits all included Swiss N1 genes (PQ098778.1, PQ098642.1, PQ098751.1) of the subtype H5N1 and the strains were isolated in 2022 and 2023 (pairwise identity set at 96.91%). The blast also revealed an important pairwise identity of 94.85% with strains isolated from cattle and milk from the USA (PV091375.1, PQ373713.1, PQ373729.2).

The recombination study was run again with the sequences obtained with the C-terminal blast (100 bp) included in the initial set tested. PP422536 and PP422535 maintained two potential breakpoints at positions [PP422536: 15 and 1374, PP422535: 29 and 1375] with the same methods (according to the reference sequence PP422536 alignment) when analyzed with RDP 4 software (Table 3). The analysis also recognized an Egyptian sequence as the major parent [OQ607880.1 (A/chicken/Egypt/F71-F114C/2022(H5N1))], and FJ032003.1 (A/mallard/ON/499/2005(H5N1)) remained as the minor parent. The pairwise identity with the major parent between the two breakpoints was comparable to the first test (48% to 99%). Regarding the minor parent, pairwise identity was mostly lower than 12% when compared to PP422536 (Figure 4.c). The second attempt also didn't determine the parental sequence of the C-terminal region.

GARD was similarly used with the same set of sequences, and two potential hotspots were detected at position 1336 (56%) and 1339 (34%) (according to the reference sequence PP422536 alignment) (AIC-$c_{baseline}$ = 9604.95, c-$AIC_{1336breakpoint}$ = 9552.5) (Figure 4.d). The raw data (chromatogram) of PP422536 carrying the potential recombination hotspots is shown in Figure 4.e.

Table 3. Data of RDP4 analyses .

Methods	Sequence carrying a potential breakpoint	*p*-values
	First attempt	
RDP	PP422536	2.986×10^{-10}
GENECONV	PP422536 PP422956 PP422535	7.197×10^{-6}
BootScan	PP422536 PP422535	2.162×10^{-7}
MaxChi	PP422536	1.529×10^{-3}
Chimaera	PP422536	1.034×10^{-2}
3Seq	PP422536	7.898×10^{-8}
	Second attempt	
RDP	PP422536	1.768×10^{-08}
GENECONV	PP422536 PP422535	6.816×10^{-5}
BootScan	PP422536 PP422535	2.242×10^{-7}
MaxChi	PP422536	2.363×10^{-2}
Chimaera	PP422536	2.268×10^{-2}
3Seq	PP422536	$1.73\ 5 \times 10^{-5}$

Stalk Characterization

Stalk deletion in NA is considered a virulence trait contributing to the high pathogenicity character within influenza viruses, and it was observed in N1, N2, N3, N5, N6, and N7 subtypes (43,44). Our isolates' N1 sequences were aligned using Clustal Omega on NGPhylogeny (45) with random sequences from isolates of each year, starting with Gs/GD strain (A/goose/Guangdong/1/1996) downloaded from the Influenza Virus database

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(https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?). The alignment was then represented on JalviewJS (RRID:SCR_006459) (46). According to Hermann et Krammer (2025) (43), the stalk deletion appeared in 2002 and remained until 2022 in H5N1 strains. However, when recreating the same flow, we observed that our isolates maintained their long stalk, and in addition, the deletion was sporadic during the period mentioned (Figure 5). The excision involved 20 aa from positions 50 to 69 (according to GS/GD strain alignment).

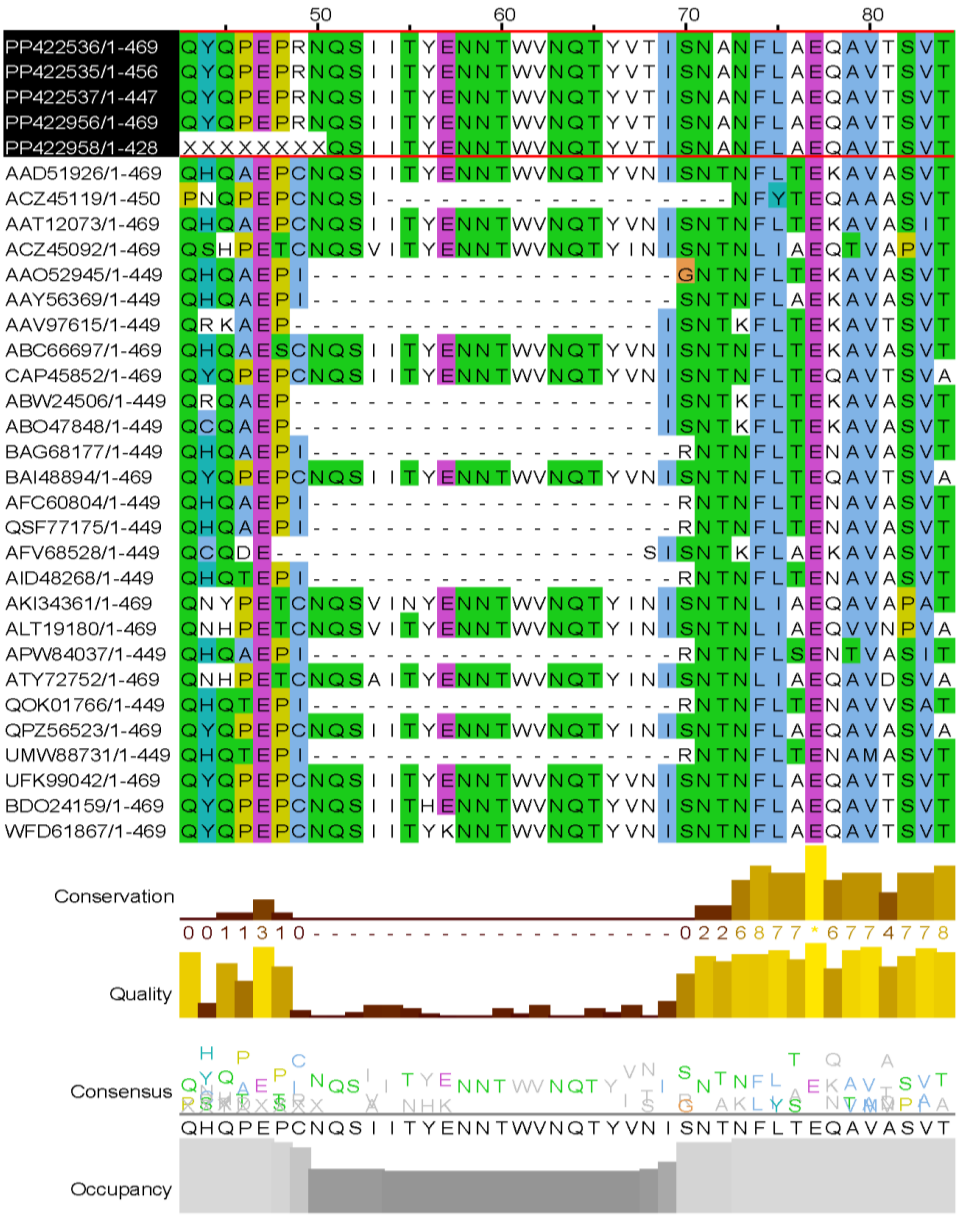

Figure 5. Alignment with N1 sequences of different timelapses. This multi-sequence alignment clearly shows the stalk deletion between positions 50 to 69 (according to the GS/GD strain alignment).

Discussion

During the two epizooties of H5N8 (2020-2021) and H5N1 (2022-2023) that affected Algerian poultry production, samples were collected from infected animals raised in different farms across the country based on disease symptoms, mortality rate, and gross lesion examination. Following RT-PCR analyses that confirmed the presence of AIV genomes of the highly pathogenic H5N1 and H5N8 strains, here we sequenced six HA-H5 and five NA-N1 from H5N1 strains and nine HA-H5 and ten

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NA-N8 from H5N8 strains. Overall, the sequencing data targeted viruses circulating in eight affected farms by the H5N8 and five others by the H5N1 strains.

Pathotyping

It is now well established that pathological AIV H5N1 and H5N8 strains isolated in Africa from migratory birds in Mauritania (47), Namibia (48), Lesotho (49), and Egypt (50,51) share the same aa sequences of the cleavage site (CS). Interestingly, the great majority of the cases that shared similar lesions like those observed in animals infected with our strains (30); and those isolated in [Nigeria (**MF112620, MW961492, MW961484, MN759490**), England (52), Serbia (**PP853096**), Netherlands (**KR233690**) and France (53)] shared the same aa composition of CS. However, heterogenous CS aa sequences were observed in the genomes of the H5N1 strains isolated in Nigeria in 2016 and France in 2015, and the H5N8 strain isolated in the Netherlands in 2014 (Figure 6). The dissimilarities in some amino acids don't seem to affect the strain's infectivity and tropism.

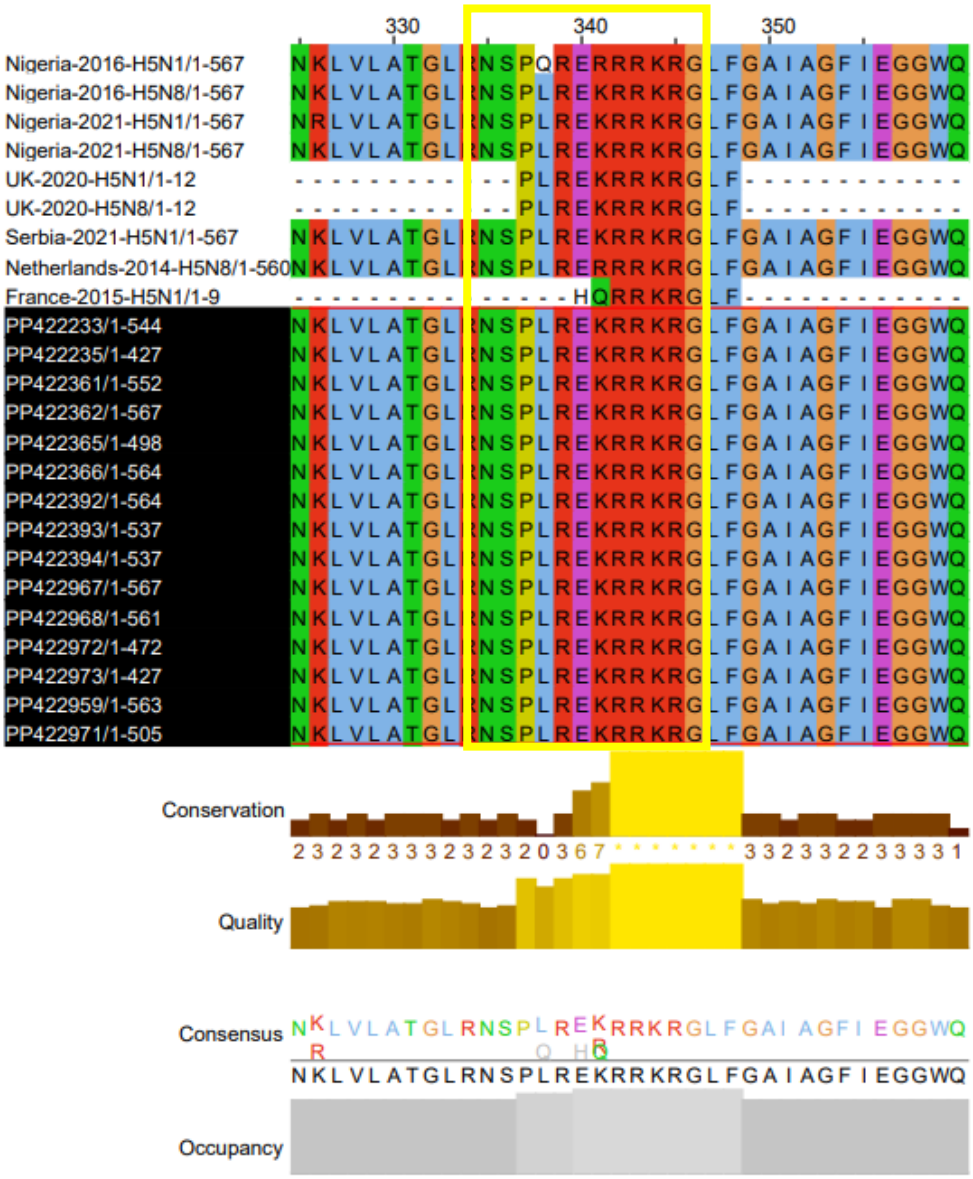

Figure 6. Sequence comparison of cleavage sites (yellow frame) of reported virus strains that induced similar gross lesions.

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Phylogenetic Analysis

The H5 (H5N8) phylogenetic tree revealed that our strain isolated in Algeria was close to that isolated in Nigeria (Chicken/Nigeria/2021(H5N8)) during the 2021 H5N8 outbreak that affected poultry. Our strain was also found to be close to a Chinese strain (Cygnus Columbianus/Hubei/2020 (H5N8)) isolated from one of the thirteen wild birds found dead in a lake in Hubei district in 2020 (54). There were also close similarities with other H5 genes, suggesting that reassortments happened before or after its transit in Algeria. The N8 gene was close to the number of isolates collected in 2020 from wild and domestic birds (France) (Kazakhstan).

Both genes of the H5N1 subtype seem related to a strain isolated in Mauritania from a dead pelican found in the National Park of Diawling with 2,140 other migratory birds (47). The outbreak was declared in February 2022, before the Algerian outbreak; therefore, it can be the same circulating strain. N1 sequences were similar to those isolated in 2022 from a black Tibetan bear in France, suggesting that the Algerian N1 might have the ability to fulfil the infection cycle within a mammal host.

Based on the sequences compared to our isolates and the migratory flyways crossing Algeria (30), the introduction of the two viruses was possible through one of the three corridors described. Regarding the H5N8 outbreak of 2020-2021, we can incriminate the East-Atlantic flyway if we consider China as the origin of the strain, which crossed Kazakhstan (West Asia) and France (Western Europe) before it arrived in Algeria. For the 2022-2023 H5N1 epizooty, it was carried through two possible flyways; therefore, if we take Egypt as the source of the studied strain, the corridor that crosses South-East Asia to reach Central America is plausible. On the other hand, if we incriminate Western Europe as the source of the strain, in this case France and Spain, migratory birds might have taken the East-Atlantic flyway before landing in Algeria and/or in Mauritania. These hypothetical origins of the Algerian strains align with the phylogenetic analysis that showed that the HA and NA studied were related to the Eurasian lineages and belonged to the 2.3.4.4b clade.

Recombination Events in the N1 Gene

As for the N1 gene, the consensus showed to be closer to the North American cluster. Further analysis was conducted to detect a recombination hotspot, and it revealed a potential breakpoint at position 1360 (according to GARD) or at 1380 (according to RDP 4). As the methods used didn't point out the parental sequence of the C-terminal region, the blast conducted showed close homology to Swiss strains isolated during the same period of the Algerian H5N1 epizooty. Homologous recombination is known to be a rare event within influenza viruses (55); however, He et al. (7) previously determined a potential breakpoint in the N1 genes of H5N1 subtype at position 1090 within Chinese strains. These events were also encountered in PA, PB1, PB2, HA, and NP segments (7, 8, 56, 57).

Long Stalk Persistence

The aa multiple alignment conducted to verify the stalk deletion in our isolates showed the conservation of these 20 aa in comparison to different N1 sequences from different years. Results also showed that the deletion was not a persistent trait in other isolates; however, it might be a predominant motif in most strains (58, 59). This kind of deletion is known to confer an increase in pathogenicity and a better adaptability in hosts (60). It is also observed when the virus is transmitted from wild to domestic birds (24), suggesting that this change is probably associated with the variation noted in the HA's CS during this host transition.

Conclusion

Highly pathogenic H5N8 and H5N1, belonging to clade 2.3.4.4b, struck Algeria and heavily damaged the poultry industry. Further molecular analysis was conducted to characterize the strain's

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origin and pathotype. The H5N8 and H5N1 hemagglutinin and neuraminidase genes were studied separately. Although the highly pathogenic character of the isolates was already established based on mortality rate and necropsy observations, the presence of the polybasic cleavage site confirms this finding. The distant and nearest origin of both the subtypes of HA and NA genes is probably Eurasian, considering the proximity noted after phylogenetic analysis. However, the N1 gene seemed to carry a recombination breakpoint in the C-terminal region, and stalk deletion was also detected. Algeria is crossed by three different migratory flyways, future AIV-H5Nx introductions are plausible mainly through Europe and Asia. The latter can't be controlled; however, dissemination across the different farms can be diminished if proper biosecurity measures and a suitable vaccination plan are applied.

Contributions: All authors contributed to the study's conception and design. A.N., K.R., and C.Y. performed PCR and semi-nested PCR analyses. A.N., C.Y., S.E.H., and G.D. performed sequencing and data analysis. A.N. wrote the initial draft of the manuscript, and then all authors contributed to generating subsequent versions to get the final document. All authors read and approved the final draft of this manuscript.

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