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Review Article

Molecular and Phylogenetic Analysis of the Highly Pathogenic Avian Influenza Virus H5N1 Detected in Mali During the 2021 and 2022 Outbreaks

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Abstract

Highly pathogenic avian influenza (HPAI) H5N1 continues to pose a significant threat to animal health, poultry production, and public health worldwide. Mali experienced two outbreaks of Highly Pathogenic Avian Influenza (HPAI) H5N1 in 2021 and 2022, raising concerns about viral evolution and potential public health risks in West Africa. Suspected samples (Tissue and swab) collected from sick and dead birds during these outbreaks were analyzed at the Central Veterinary Laboratory of Bamako using quantitative real-time PCR. Whole-genome sequencing was performed on thirteen H5N1-positive samples using the Illumina MiSeq platform with a paired-end sequencing approach. Phylogenetic analyses were conducted using the maximum-likelihood method implemented in IQ-TREE v1.6.6. Phylogenetic analysis of the hemagglutinin (HA) gene revealed that all detected viruses belonged to clade 2.3.4.4b and clustered closely with strains previously reported in West Africa. In-depth analysis of the whole genome phylogenetic topology revealed the emergence of an H9N2/H5N1 reassortant strain during the 2022 outbreak. Molecular characterization of all eight genomic segments identified several mutations associated with host specificity, including markers linked to adaptation in gallinaceous poultry and mutations potentially associated with increased zoonotic potential. These findings demonstrate the continued genetic evolution of H5N1 viruses circulating in Mali and emphasize the importance of sustained genomic surveillance. Strengthening hygiene practices and biosecurity measures in poultry farms, live bird markets, and slaughterhouses is essential to reduce the risks posed to both animal and public health.

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Keywords

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1. Introduction

Avian influenza (AI), caused by viruses that belong to the family Orthomyxoviridae, genus Alphainfluenzavirus (Influenzavirus A or influenza A virus), is a zoonotic disease that can infect several species of domestic birds (*Gallus gallus domesticus*, Meleagris, *Coturnix coturnix, Numididae*, etc.) as well as wild aquatic birds which constitute the natural reservoir of the virus. These subtypes, the A/Goose/Guangdong/1/1996 (Gs/GD) lineage A (H5N1) viruses are currently the most widespread of the HPAI strains in bird populations [1]. Since its emergence in 1996, the hemagglutinin gene (HA) of the Gs/GD-like A (H5N1) has continually evolved both through accumulation of mutations which have resulted in the emergence of multiple HA genetic clades and subclades [2]. By infecting wild birds and exploiting their migratory routes, multiple clades of the GS/GD lineage have been responsible of five intercontinental epidemic waves, which resulted also in multiple introductions of the virus in the African continent. Since 2021, a highly pathogenicity strain of avian influenza known as HPAI H5N1 clade 2.3.4.4b has caused a panzootic event of unprecedented proportion for the avian population and has been involved in several spill over events in aquatic and terrestrial mammals as well as sporadic infections in humans [3]. Avian influenza viruses represent a major threat to human and animal health. These viruses are responsible for high morbidity and mortality, especially in the poultry industry, resulting in huge economic losses since its first introduction in the west African region in February 2006 [4, 5].

The poultry industry in Mali is characterized by the traditional or village poultry farming system estimated at more than 90% of the poultry population. It is present in the majority of rural families and plays a vital role in the revenue diversification and socio -cultural events. The semi-industrial or modern poultry farming system is mainly located in the peri-urban areas of the main cities such as Bamako, Segou, Sikasso, Kayes, etc. The poultry industry by its socio-economic impacts is essential in national economic growth and in reducing food insecurity and poverty in Mali, according to the National Directorate of Productions and Animal Industries Report (Mali. D. N. P. I. A report, 2023).

Mali has faced its first Highly Pathogenic Avian Influenza (HPAI) H5N1 epizootic in March 2021. A dozen poultry farms were affected in the peri-urban areas of Bamako and the nearest city Koulikoro with more than 54,147 deaths. Unfortunately, in 2022 at the same period, in March, the disease reappeared in the same areas plus one more city in the southern part of the country, Sikasso with more than 3,58,513 deaths according to the National Directorate of Veterinary Services (Mali. D. N. S. V report, 2022).

2. Materials and Methods

2.1. Samples Collection

Between March and April of 2021 and 2022, high poultry mortality was reported in commercial farms located in the peri-urban areas of Bamako, Kati, and Sikasso (Figure 1) to the veterinary services of Bamako. Following these reports, field investigations were conducted, and random sampling was performed. Cloacal and tracheal swabs were collected from clinically affected broilers and laying hens, and placed in 2 mL cryovials containing 1 mL of virus transport medium (PAN VTM HBSS, 1 L; No. P04-01754).

Tissue samples were additionally collected during necropsy of euthanized or dead birds at the Central Veterinary Laboratory (CVL, Bamako, Mali). A total of 28 samples (including tissue, cloacal swabs, and tracheal swabs) were received 2021 and 31 in 2022.

2.2. RNA Purification and Amplification

Total RNA was extracted from 100 µL of each sample using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Detection of influenza A virus was performed by one-step quantitative real-time RT-PCR (qRT-PCR) using the AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA, USA), targeting the matrix (M) gene, on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Munich, Germany [6].

Samples that tested positive for the M gene were further subtyped for H5N1 using a duplex one-step real-time qRT-PCR assay as previously described [6, 7].

A subset of M gene-positive samples, including 12 from 2021 (tissue homogenates and swabs) and 14 from 2022, were submitted to the Food and Agriculture Organization /World Organization for Animal Health (FAO/WOAH) reference laboratory (Istituto Zooprofilattico Sperimentale delle Venezie, IZSVe, Italy) for genomic sequencing and further characterization.

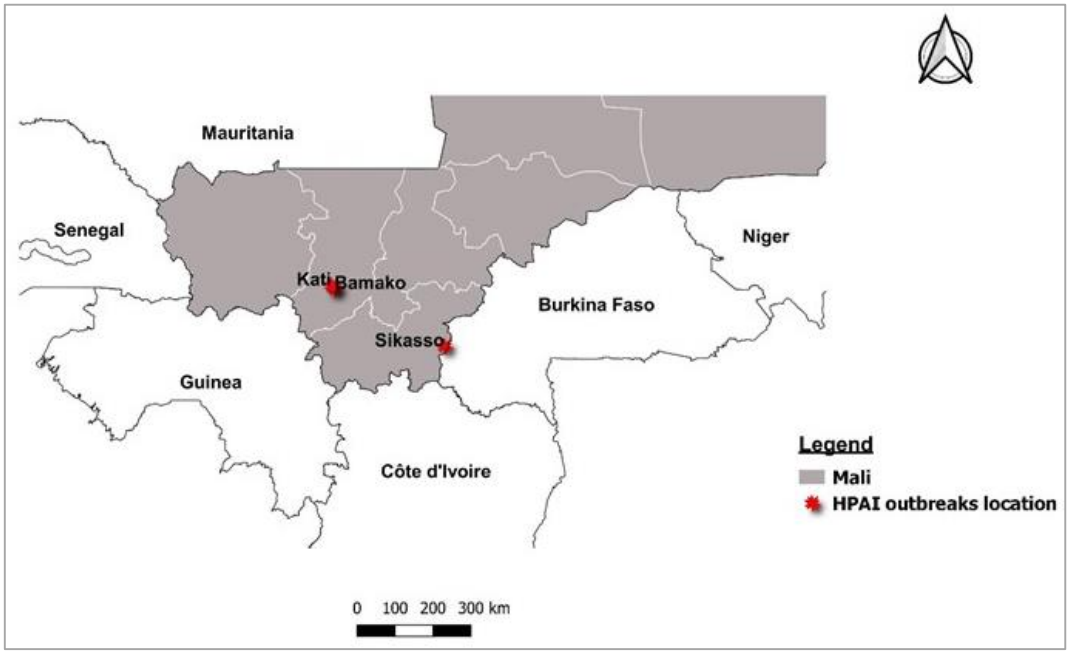


Figure 1. *Regions where the 2021 and 2022 HPAI outbreaks occurred in Mali.*

2.3. Sequencing and Sequences Analysis

A target RT-PCR approach was used to amplify influenza A virus whole genomes from positive clinical specimens as previously described [1]. Sequencing libraries were obtained using the Illumina DNA prep kit (Illumina, San Diego, CA, USA). The amplicons were sequenced on Illumina's MiSeq instrument with a 2x 250 PE mode using the protocol and reagents of the MiSeq v2 kit of 300 cycles (2 ×150bp) according to the manufacturer's instructions.

The raw sequencing reads produced by the MiSeq instrument were cleaned with Trimmomatic v0.32 with minimum quality 20. Illumina DNA prep adapter sequences were clipped from reads using scythe v0.991 (https://github.com/vsbuffalo/scythe) and amplification primers were removed using sickle v1.33 (https://github.com/najoshi/sickle) Read shorter than 80bp or unpaired were discarded. The cleaned reads were aligned against a reference genome using the MEM algorithm from BWA v0.7.12-r1039. Picard tools v2.1.0 (http://broadinstitute.github.io/picard/) and GATK v3.530-32 [8] were used to improve alignment quality, correct potential errors and recalibrate base quality score. LoFreq v2.1.2.33 [9] was used to call single nucleotide polymorphisms which were reported in a vcf file. The generated vcf file was 2 then used to produce the consensus sequences using an in-house script. Briefly, this script calls the base for each position with a coverage >10X, considering all the polymorphisms with a frequency higher than 25%. "N" is assigned to all positions with a coverage lower than 10 reads.

Consensus sequences of the eight gene segments were aligned with the most related sequences available in Global Initiative on Sharing All Influenza Data (GISAID) using MAFFT v. 7 [10]. Maximum likelihood (ML) phylogenetic trees were obtained for each gene using IQTREE v1.6.6, performing ultrafast bootstrap resampling analysis (1,000 replications) and using the best-fitted nucleotide substitution model selected by ModelFinder [11-13].

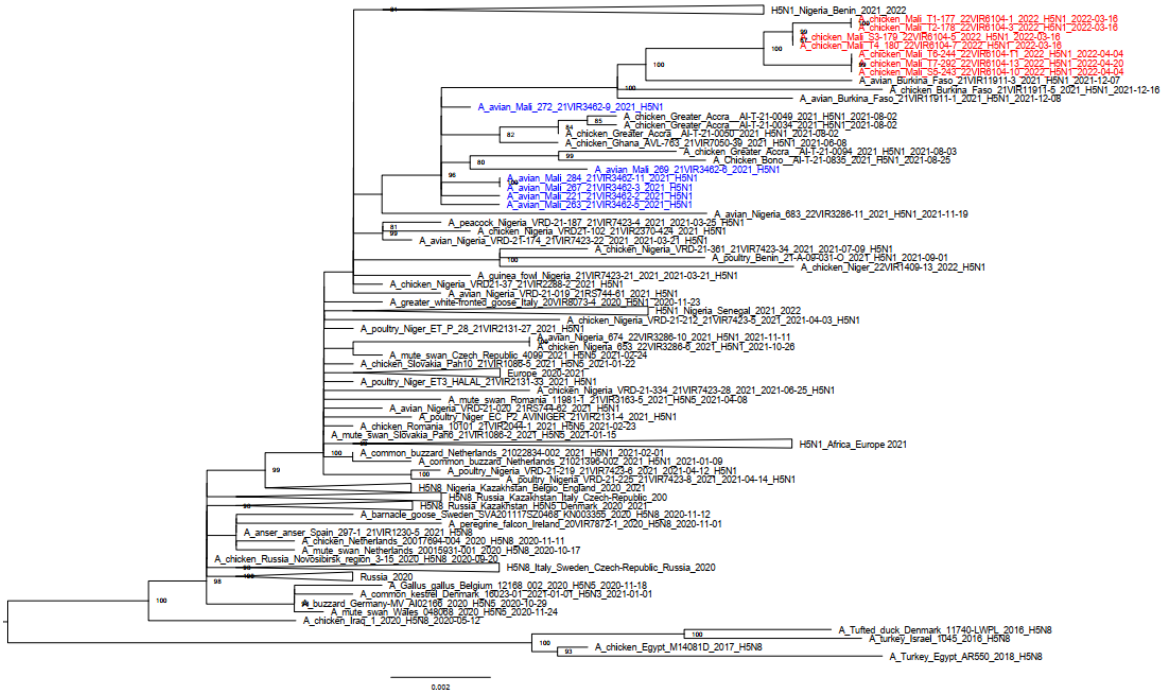
3. Results

3.1. Pathogen Identification and Characterization

All the tissue and tracheal/cloacal swabs samples from 2021 and 2022 tested by rRT-PCR were positive (with a high Cycle Threshold value (CT) value between 22-26) for the HPAIV H5N1.

The analysis of the complete hemagglutinin (HA) gene segment shows that all the HPAI H5N1 viruses identified in Mali between March/April 2021, March/April 2022 belong to the clade 2.3.4.4B and cluster together with the HPAI H5N1 viruses collected from poultry in other Western African countries, namely, Nigeria and Niger in 2021-2022 (Figure 2). An in-depth analysis of the HA topology indicates that the potential offspring of this genetic cluster can be traced back to viruses identified in the European continent starting from late 2020.

The analysis of the other gene segments confirms the clustering of the HA gene except for the PA segment where the 2022 H5N1 viruses from Mali group together with H9N2 viruses collected in West Africa between 2017 and 2020 and with H5N1 viruses identified in Burkina Faso previously described as H5N1/H9N2 reassortant strains.



(a) HA gene.

(b) PA gene

Figure 2. *Maximum likelihood phylogenetic trees of the hemagglutinin (HA) and polymerase acidic (PA) genes.*

(a) Represents the maximum likelihood phylogenetic tree of the complete hemagglutinin (HA) gene segment shows in blue, the 2021 H5N1 viruses from Mali cluster with other H5N1 viruses identified in Ghana and Ivory Coast in August 2021; in red the 2022 H5N1 viruses from Mali which cluster with other H5N1 identified in Burkina Faso in December 2021. (b) While the phylogenetic analysis of the complete polymerase acidic (PA) gene shows in blue, the 2021 H5N1 viruses from Mali cluster with other H5N1 from Ghana, Nigeria and Benin. H5N1 viruses from Mali 2022 belong to another branch of the tree and cluster with H9N2 viruses from Burkina Faso.

These findings indicate that H5N1 viruses from Mali 2022 are H5N1/H9N2 reassortant strains for the gene PA.

The phylogenetic tree was constructed using IQTREE v1.6.6, with only Bootstrap values greater than or equal to 80 reported at the node level.

3.2. Detection of Mutations Associated with the Specificity and/or Pathogenicity of the Avian Influenza Virus

Table 1. *Mutations Identified in 13 H5N1 Highly Pathogenic Avian Influenza Viruses from Mali (2021-2022).*

Marker	Effects	H5N1 viruses from Mali	Citation
PB2 gene			
V598T	Increased virulence in mice; Increased polymerase activity in mammalian cells; Increased replication in mammalian cells	All	Hu M. et al., (2017); Suttie A. et al., (2019)
S715N	Decreased virulence in mice	All	Sun H. et al., (2015); Suttie A. et al., (2019)
L89V; G309D; T339K; R477G; I495V; K627E; A676T	Increased polymerase activity in mammalian cells; Increased virulence in mice	All but: Mali/221_21VIR3462-2/2021. Mali/263_21VIR3462-5/2021	Li J. et al., (2009); Suttie A. et al., (2019)
L89V; G309D	Increased polymerase activity in mammalian cells; Increased virulence in mice	All but: Mali/221_21VIR3462-2/2021	Li J. et al., (2009); Suttie A. et al., (2019)
K526R	Increased polymerase activity in mammalian cells	Only 2022	Song W. et al., (2014); Suttie A. et al., (2019)
K389R	Increased polymerase activity in mammalian cells; Increased replication in mammalian cells	All but: Mali/221_21VIR3462-2/2021	Hu M. et al., (2017); Suttie A. et al., (2019)
E627K	Increased virulence in mice; Decreased replication in avian cells; Enhanced polymerase activity; Decreases virulence in chickens; Decreased polymerase activity in avian cells; Contributes to contact transmission in guinea pigs; Contributes to airborne pathogenicity in ferrets	Only: Mali/263_21VIR3462-5/2021	Fornek J. L. et al., (2009); Herfst S. et al., (2012); Le Q. M. et al., (2005); Hatta M. et al., (2007); Bortz E. et al., (2011); Hatta H. et al., (2001); Richard M. et al., (2017); Shinya K. et al., (2004); Manzoor R. et al., (2009); Chen H. et al., (2007); Mase M. et al., (2006); Bogs J. et al., (2011); Kim J. H. et al., (2010); Long J. S. et al., (2013); Suttie A. et al., (2019)
PB1 gene			
N66S	Enhanced replication in mice; Enhanced virulence in mice; Enhanced antiviral response in mice	All	Conenello G. M. et al., (2007); Schmolke M. et al., (2011); Suttie A. et al., (2019)
D622G	Increased polymerase activity in mice; Increased virulence in mice	All	Feng X. et al., (2016); Suttie A. et al., (2019)
D3V	Increased polymerase activity in avian cells; Increased replication in avian cells; Increased polymerase activity in mammalian cells; Increased replication in mammalian cells	All	Elgendy E. M. et al., (2017); Suttie A. et al., (2019)
PA gene			
S37A	Increased polymerase activity in mammalian cells	All	Yamayoshi S. et al., (2014); Suttie A. et al., (2019)
Q400P	Decreased virulence in mice	Only 2022	DesRochers B. L. et al., (2016); Suttie A. et al.,

Marker	Effects	H5N1 viruses from Mali	Citation
			(2019)
P190S	Decreased virulence in mice	All	DesRochers B. L. et al., (2016); Suttie A. et al., (2019)
N409S	Increased polymerase activity in mammalian cells; Increased replication in mammalian cells	Only 2021	Yamayoshi S. et al., (2014); Suttie A. et al., (2019)
N383D	Increased polymerase activity in mammalian cells; Increased polymerase activity in avian cells	All	Song J. et al., (2015); Song J. et al., (2011); Suttie A. et al., (2019)
HA gene			
Marker H5 Numbering			
K64E	Increased pH of fusion; Decreased HA stability; Decreased virulence in mice	All	Sun X. et al., (2019); Suttie A. et al., (2019)
V182N	Increased virus binding to α2-6; Decreased virus binding to α2-3	All	Lu X. et al., (2013); Suttie A. et al., (2019)
S154N	Increased virus binding to α2-6	All	Wang W. et al., (2010); Suttie A. et al., (2019)
S133A	Increased pseudovirus binding to α2-6	All	Yang Z. Y. et al., (2007); Suttie A. et al., (2019)
S107R; T108I	Increased virulence in chickens; Increased virulence in mice; Increased pH of fusion	All	Wessels U. et al., (2018); Suttie A. et al., (2019)
K218Q; S223R	Increased virus binding to α2-3; Increased virus binding to α2-6	Allk	Guo H. et al., (2017); Suttie A. et al., (2019)
NP gene			
M105V	Increased virulence in chickens	All	Tada T. et al., (2011); Tada T. et al., (2011); Suttie A. et al., (2019)
A184K	Increased replication in avian cells; Increased virulence in chickens; Enhanced interferon response	All	Wasilenko J. L. et al., (2009); Suttie A. et al., (2019)
MP gene			
T215A	Increased virulence in mice	All	Fan S. et al., (2009); Suttie A. et al., (2019)
N30D	Increased virulence in mice	All	Fan S. et al., (2009); Suttie A. et al., (2019)
I43M	Increased virulence in chickens; Increased virulence in ducks; Increased virulence in mice	All	Nao N. et al., (2015); Suttie A. et al., (2019)
NS gene			
V149A	Increased virulence in chickens; Decreased interferon response in chickens	All	Li Z. et al., (2006); Suttie A. et al., (2019)
P42S	Increased virulence in mice; Decreased antiviral response in mice	All	Jiao P. et al., (2008); Suttie A. et al., (2019)
L103F; I106M	Increased virulence in mice	All	Kuo R. L. et al., (2009); Spesock A. et al., (2011); Suttie A. et al., (2019)
I106M	Increased viral replication in mammalian cells; Increased virulence in mice	All	Ayllon J. et al., (2014); Suttie A. et al., (2019)
C138F; K55E; K66E	Enhanced replication in mammalian cells; Decreased interferon response	All	Li J. et al., (2018); Suttie A. et al., (2019)
C138F	Increased viral replication in mammalian cells; Decreased interferon response	All	Li J. et al., (2018); Suttie A. et al., (2019)

The results showed that all the HPAI H5N1 viruses detected in Mali during the 2022 outbreaks present more mutations than those detected in 2021 (Table 1) which only have one on the hemagglutinin (HA) protein as well as a deletion on the neuraminidase (NA) protein which is also found in the 2022 viruses.

4. Discussions

The findings of the present study demonstrated that the HPAI H5N1 viruses detected in Mali in 2022 differ substantially from the virus identified in 2021. Several mutations identified in the viral genomes have previously been described as markers of gallinaceous host specificity or as determinants associated with reduced antiviral responses in chickens [14]. However, additional experimental investigations are required to clarify the phenotypic significance of these mutations.

Phylogenetic analysis of the complete hemagglutinin (HA) gene segment (Figure 1), together with all other gene segments except polymerase acidic (PA), showed that the HPAI H5N1 viruses detected in Mali in 2022 clustered with the virus identified in 2021 and with H5N1 viruses detected in Europe between 2020 and 2022. These findings agree with results reported from Senegal during the same period, where phylogeographic analysis suggested southward spread of the virus from southern Europe into West Africa. Collectively, these observations support the hypothesis that West Africa may serve as an ecological sink for HPAI H5N1 viruses circulating in Europe [15].

In contrast, analysis of the PA gene demonstrated that the 2022 Malian viruses were reassortant H5N1/H9N2 strains closely related to those previously identified in Burkina Faso (Figure 2). This finding suggests that viruses initially introduced from Europe continue to evolve within West African poultry populations, likely driven by the endemic circulation of H9N2 viruses among domestic birds. Nevertheless, the currently available data do not allow precise determination of where this reassortment event occurred.

Notably, H9N2 viruses were detected in commercial poultry farms located in the peri-urban area of Bamako during March-May 2021 and again during March-May 2022, which may partly explain the emergence of the reassortant viruses. The close similarity between reassortant strains identified in Mali in 2022 and those detected in Burkina Faso in late 2021 further suggests a common origin of the outbreaks in the two countries. This observation points to the emergence of a new hotspot for H5N1/H9N2 virus spread in West Africa. It also highlights how porous borders in the region may facilitate transboundary dissemination of HPAI viruses [16].

Previous studies have also documented the circulation of H9N2 viruses in poultry markets in Mali [17]. Control efforts in the country rely mainly on passive surveillance in poultry markets, weekly fairs, and both traditional and commercial farms, generally following reports of unusually high mortality (Mali D. N. S. V. report, 2023). Several poultry trade-related factors may contribute to the persistence and spread of avian diseases such as avian influenza (AI) and Newcastle disease (ND). These include the transport of live birds to and from poultry markets, the mixing of multiple domestic bird species and occasionally wild birds, and prolonged holding times, often exceeding one day, all of which may favor viral dissemination and genetic reassortment [18].

Illegal trade in live poultry and poultry products across porous borders in West Africa, together with inadequate biosecurity and hygiene measures in poultry farms and markets, may also contribute to the introduction and spread of these viruses in Mali [17]. In addition, previous studies have suggested that migratory birds may play a role in the initial introduction of HPAI H5N1 viruses [19, 20]. In Mali, the 2021 and 2022 outbreaks were recorded between February and March, corresponding to the period during which Eurasian migratory birds reach West Africa [20].

Although clade 2.3.4.4 HPAI H5Nx viruses are characterized by substantial reassortment capacity [21], reassortment events involving H9N2 viruses remain rarely reported despite the extensive co-circulation of both virus groups in poultry populations in Asia and Africa. Most naturally occurring clade 2.3.4.4 H5Nx/H9N2 reassortants have been identified in domestic birds in China (2015, 2016) [22, 23] and Egypt (2018, 2019) [24, 25]. Importantly, clade 2.3.4.4 HPAI H5N6 viruses carrying internal gene constellations derived from H9N2 viruses were also reported to infect humans in China in 2015 and 2016 [26], highlighting the potential public health relevance of such reassortment events.

5. Conclusion

In conclusion, the HPAI H5N1 viruses detected in Mali in 2021 were genetically distinct from those identified during the 2022 outbreaks, which showed substantial evolution through reassortment and mutation accumulation. These genetic changes may have influenced viral adaptation to gallinaceous hosts and altered pathogenicity. The detection of H9N2-derived gene segments further illustrates the dynamic evolutionary potential of avian influenza viruses and their persistent threat to animal and public health. The emergence and spread of these reassortant strains pose a significant risk to poultry production, food security, and livelihoods. These findings emphasize the need for strengthened surveillance in live bird markets, weekly fairs, traditional and intensive poultry farms, and wild birds, as well as enhanced subregional collaboration for epidemiological data sharing and genetic monitoring of avian influenza viruses in West Africa.

Abbreviations

AI	Avian Influenza
BWA	Burrows-Wheeler Aligner

CT	Cycle Threshold
CVL	Central Veterinary Laboratory
DNA	Deoxyribonucleic Acid
DNSV	National Directorate of Veterinary Services
DNPIA	National Directorate of Productions and Animal Industries
ECTAD	Emergency Center for Transboundary Animal Diseases
FAO-UN	United Nations -Food and Agriculture Organizations
FAO	Food and Agriculture Organization
GATK	Genome Analysis Toolkit
GISAID	Global Initiative on Sharing All Influenza Data
HA	Hemagglutinin Gene
HPAI	Highly Pathogenic Avian Influenza
M gene	Matrix Gene
MAFFT	Multiple Alignment Using Fast Fourier Transform
ML	Maximum Likelihood
ND	Newcastle Disease
PA	Polymerase Acidic
PCR	Polymerase Chain Reaction
PE	Paired-end
qRT-PCR	Quantitative Reverse Transcription Pol Ymerase Chain Reaction
RAF	Regional Office for Africa
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
WOAH	World Organization for Animal Health

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Cheick Abou Kounta Sidibe: Resources, Supervision, Validation, Writing – review & editing

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Isabella Monne: Data curation, Formal Analysis, Methodology, Software, Supervision, Validation, Visualization, Writing – review & editing

Antoine Dara: Data curation, Formal Analysis, Methodology, Software, Supervision, Validation, Visualization, Writing – review & editing

Conflicts of Interest

The authors declare that they have no known conflicts of interest or personal relationships that could have appeared to influence the work reported in this paper.

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