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Paper

Molecular Detection and Genetic Characterization of H9N2 Avian Influenza Virus in Laying Hen and Broiler Farms in Dakar and Thiès Regions, Senegal

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Abstract

Avian influenza, particularly the low pathogenic H9N2 subtype, is a major challenge to poultry and public health in Senegal. As important as it is, little is known about the occurrence and genetic characteristics of the H9N2 detected within the country's poultry farms. This study was therefore carried out to assess the occurrence and the molecular characteristics of H9N2 avian influenza virus strains in broiler and layer farms of the Dakar and Thiès regions. Throughout a period of 16 months, a cross-sectional survey was undertaken, collecting 390 tracheal and cloacal swabs from 39 chicken farms that reported respiratory disease. Viral RNA was isolated and analysed using real-time RT-PCR followed by viral isolation and hemagglutinin gene sequencing on positive samples. Phylogenetic analysis was performed to determine genetic associations with regional strains. Overall, the prevalence was 9.2% across 87 pools made from 390 swabs, with broiler chickens showing a high prevalence of 19.4% and layer hens a low prevalence of 2.0%. The majority of the positive farms were located in the Thiès region, which accounted for 6 out of the 8 positive farms. Phylogenetic analysis revealed that the circulating strain belonged to the G1 lineage and was closely related to Moroccan strains, indicating likely regional spread through poultry trade. The strains did not possess any molecular markers of increased virulence or zoonotic potential. These findings confirm the endemicity of H9N2 in Senegal's important poultry-producing regions and raise the need for targeted surveillance and improved biosecurity to avoid outbreaks and preserve public health.

Keywords

Poultry, Low pathogenic H9N2 avian influenza virus, Molecular characterization, Poultry disease surveillance, Senegal

Introduction

Avian influenza is a highly contagious disease caused by viruses belonging to the *Orthomyxoviridae* family. These are segmented, single-stranded, negative-sense RNA viruses classified into four types: A, B, C, and D . Among these, type A influenza viruses are the most relevant to poultry health due to their diversity and their ability to infect a wide range of avian species . Type A viruses are further categorised highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI), based on their ability to cause disease in chickens . HPAI viruses typically cause acute, systemic disease with high mortality rates in chickens and other *galliform* birds, whereas LPAI viruses generally result in mild respiratory or reproductive infections . Among the LPAI viruses, the H9N2 subtype is of particular concern. It is the most distributed LPAI virus globally and has become endemic in various regions, such as North and West Africa. Since 2017, H9N2 viruses have been identified in several West African countries such as Benin, Ghana,

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Togo, Nigeria, and Senegal . Although less virulent than HPAI viruses, H9N2 infections can significantly impair egg production and growth performance, leading to substantial economic losses in the poultry sector. Moreover, H9N2 viruses are zoonotic, with occasional transmission to humans, and they pose a risk of genetic reassortment, potentially giving rise to more pathogenic strains . In Senegal, the poultry industry represents a major component of the national agricultural economy. It plays a vital role in food security, poverty reduction, job creation, women's empowerment, and education . Following the government's ban on poultry importation in 2005, domestic production has expanded significantly, reaching over 135,000 tons of poultry meat and 1,5 billion eggs in 2021, with revenues exceeding 450 billion CFA francs . However, this growth has also intensified the risk of infectious disease outbreaks**,** including avian influenza, which now threatens the productivity and sustainability of the sector. After the ban on poultry importation, Senegal reported its highly pathogenic avian influenza (HPAI) in 2021, initially in wild pelicans at the Djoudj National Bird Park, followed by outbreaks in commercial farms near the Pout area, with a total of five confirmed outbreaks . Despite the severity of these incidents, avian influenza surveillance in Sub-Saharan African countries remains limited in scope, and while some studies have reported the presence of H9N2 viruses in Senegal (, comprehensive data on their epidemiology and genomic diversity are still scarce. This knowledge gap hinders the implementation of targetedcontrol strategies and poses ongoing threats to both animal and human health. This study aims to assess the prevalence and molecular characteristics of H9N2 avian influenza virus strains in broiler and layer farms located in the Dakar and Thiès regions of Senegal. It was guided by key research questions: i) What is the prevalence of H9N2 in the poultry farms in these regions? ii) What is the geographical distribution of farms affected by LPAI H9N2? and iii) What are the genetic profiles and circulation patterns of H9N2 viruses in these poultry populations? The findings are expected to fill critical knowledge gaps and support the development of more effective surveillance and control measures for avian influenza in Senegal.

Materials and me thods

Description of study sites

This study was carried out in Senegal's Dakar and Thiès regions, which were selected due to their significant contributions to the country's poultry industry. Dakar, covering roughly 550 km^2 and located at 14.6928° N latitude and 17.4467° W longitude , had a population of over 3.7 million in 2019. The region is divided into four departments: Dakar, Pikine, Guediawaye, and Rufisque. Its urban setting, favourable climate, and well-established infrastructure create an ideal environment for poultry farming. Thiès, situated at 14.7901° N latitude and 16.9241° W longitude , spans a larger area of about 6,882 km^2 and had just over 2 million residents as of 2019. Dakar and Thiès are suitably adapted for poultry production due to their favourable climatic conditions. They share a contiguous border, which facilitates trade and poultry movement between the two regions. This geographical proximity, along with their combined contribution of roughly 80% to national poultry production , makes them ideal for regional comparative surveillance.

Study Design

A cross-sectional study based on passive surveillance was conducted in the Dakar and Thiès regions of Senegal. Veterinary clinics across the study areas were instructed to promptly inform the Inter-State School of Veterinary Science and Medicine of Dakar whenever farms reported chickens exhibiting respiratory disease symptoms. Following post-mortem examinations, samples were collected from birds showing lesions characteristic of avian influenza infection. On each suspected farm, 10 birds displaying severe respiratory signs such as wheezing, sneezing, and laboured breathing often accompanied by reduced feed and water intake, were selected for sampling. In collaboration with private veterinarians, a total of 390 tracheal and cloacal swabs, as well as organ samples (including trachea, lungs, kidneys, and caecal tonsils), were collected from 39 poultry farms, comprising 14 farms in Dakar (5 broiler and 9 layer farms) and 25 farms in Thiès (11 broiler and 14 layer farms). The samples were collected using Whatman® FTA® Classic Cards for better preservation and transport of nucleic acids. Although organ samples were collected during necropsy for general diagnostic purposes, only the tracheal and cloacal swabs preserved on FTA cards were used for molecular analysis, viral isolation, and sequencing in this study. Sample collection was carried out over approximately 16 months, from February 2023 to July 2024. The FTA cards were carefully labelled and transported to the Inter-State School of Veterinary Science and Medicine (EISMV)'s Microbiology, Immunology, and Infectious Pathology Laboratory. Upon arrival, the samples were stored at -20°C for further molecular analysis, viral isolation, and sequencing.

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Laboratory Analysis Procedures

Viral RNA Extraction

Small discs of 2 mm diameter were excised from each Whatman® FTA® Classic Card using a Whatman™ Harris Uni-Core™ Punch (Cytiva, UK). The discs that were collected from tracheal or cloacal swabs obtained from the same farm, were then pooled in batches of four to five samples in a laminar flow hood. Each of the pools was dissolved in 800 µL of phosphate-buffered saline (PBS) and shaken well using a vortex mixer. Following pooling, viral RNA was purified using the NucleoSpin® RNA Virus kit (Macherey-Nagel, Düren, Germany, cat. no. 740956.250) according to the manufacturer's protocol.

Real-Time RT-PCR

Detection of the H9N2 low pathogenic avian influenza virus (LPAIV) was performed using the iTaq™ Universal One-Step RT-qPCR kit (Bio-Rad, Hercules, USA), which allows for reverse transcription and amplification in a single reaction. Each reaction was carried out in a final volume of 25 µL, comprising 5 µL of extracted viral RNA and 20 µL of master mix, according to the manufacturer's instructions. Both positive and negative controls were incorporated in each run to ensure the specificity and reliability of the amplification reaction. Amplification was carried out using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), an instrument with the potential for real-time RNA target detection and quantification. The assay used primers and a TaqMan probe targeting a conserved region in the HA2 subunit of the haemagglutinin (HA) gene, as described by , to allow generic detection of H9 subtype viruses. The thermal cycling process entailed a reverse transcription at 50°C for 10 minutes, initial denaturation at 95°C for 1 minute, and 40 cycles of amplification. The process achieved specific and sensitive detection of H9N2 viral RNA in clinical specimens.

H9N2 Virus Isolation

Only the samples that tested positive by real-time RT-qPCR were employed for viral isolation to obtain a high viral load suitable for conventional RT-PCR detection. Specific-pathogen-free (SPF) 10-day-old embryonated chicken eggs were infected with a combination of 0.2 mL of viral suspension, 0.6 mL of sterile phosphate-buffered saline (PBS), and 0.2 mL of OXY-Kel 20 L.A. antibiotic (oxytetracycline) (WOAH, 2021). The inoculum was delivered into the allantoic cavity of the eggs using a sterile needle through the air sac. Following inoculation, the eggs were incubated at 37°C and examined daily for five days to assess embryo viability . Dead embryos were examined for characteristic lesions, and allantoic fluids were harvested, centrifuged to clarify them, and kept at -20°C until use. Viral RNA was then extracted the highest viral load allantoic fluid samples using the NucleoSpin® RNA Virus kit (Macherey-Nagel, Düren, Germany, Cat. No. 740956.250).

Hemagglutinin gene sequencing of Senegalese Isolates

Viral RNA isolated in the previous step was reverse transcribed into complementary DNA (cDNA) using the Applied Biosystems kit. The cDNA isolated was amplified by PCR with the DreamTaq Green PCR Master Mix. The primers described by Hoffmann et al. (2001) were used for HA gene amplification with a predicted product size of approximately 670 bp on a 1% agarose gel . All amplicons corresponding to this size were excised and purified from the gel using a Machery-Nagel kit (Nucleospin Gel and PCR Clean-up), following the manufacturer's instructions. Sequencing was carried out at Eurofins Genomics Europe Shared Services GmbH, Germany according to the manufacturer's protocol using 3130xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, USA), along with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA; Cat. No. 4337455).

Data Analysis

Descriptive statistics were used to report the occurrence of H9N2 in Dakar and Thiès' chicken flocks. All the results, including the occurrence rates and genetic comparisons, were presented in tables and figures for clarity. Genetic analysis was done using MEGA 6. Multiple sequence alignment (MSA) and comparison of sequences were carried out using BioEdit and ClustalW software , as implemented in the MEGA 6 program package by , for identifying nucleotide variations and conserved regions. The sequences were compared and aligned to the whole collection of available H9 haemagglutinin gene sequences recorded in the GenBank database of the National Centre for Biotechnology Information (NCBI) using the BLAST tool . Phylogenetic reconstruction was conducted using the Maximum Composite Likelihood method with 1,000 bootstrap replicates, found in the MEGA software package (Molecular Evolutionary

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Genetics Analysis).

Results

Occurrence of H9N2 Avian Influenza Among Broiler and Layer Farms in the Dakar and Thiès Regions

A total of 390 tracheal and cloacal swabs were tested, which were grouped into 87 pools. Out of these pools, eight were found to be positive for the H9N2 avian influenza virus, with an overall pool-level prevalence of 9.2%. Stratified by poultry type, the broiler chickens showed a high prevalence of 19.4% (7 positive pools out of 36 pools tested), whereas layer hens showed a very low prevalence of 2.0% (1 positive pool out of 230 tested) (Table 1).

Poultry Type	Samples Tested	Pools Tested	Positive Pools	Pool-level Positivity (%)
Broilers	160	36	7	19.4%
Layers	230	51	1	2.0%
Total	**390**	87	**8**	**9.2%**

Table II. Prevalence of LPAI H9N2 Among Broiler and Layer Poultry in the Dakar and Thiès Regions

Out of 39 poultry farms tested in Dakar and Thiès, 8 farms (20.5%) were positive for H9N2 avian influenza virus. Among broiler farms, 44% (7 of 16) were positive, while only 4.35% (1 of 23) of layer farms tested positive (Figure 1).

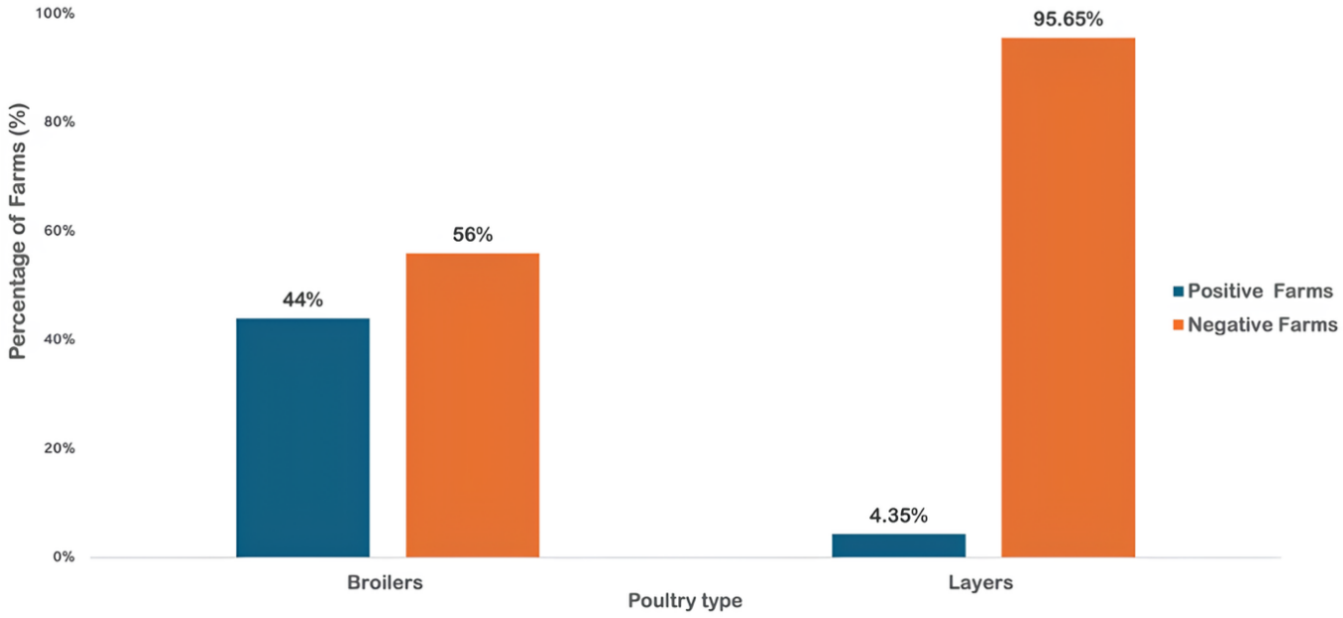

Figure 1. Distribution of H9N2 Avian Influenza based on the type of poultry across Farms in the Dakar and Thiès Regions of Senegal in 2024.

Geographical Distribution of Farms Suspected of H9N2 Infection in the Dakar and Thiès Regions of Senegal in 2024

During the study period, 39 farms including 23 layer farms and 16 broiler farms were suspected of H9N2 avian influenza virus infection based on post-mortem examinations of carcasses originating from these farms. RT-PCR results revealed that the majority (75%) of the positive farms were located in Thiès region, which accounted for 6

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farms out of the 8 positive farms, as shown on the map below (Figure 2).

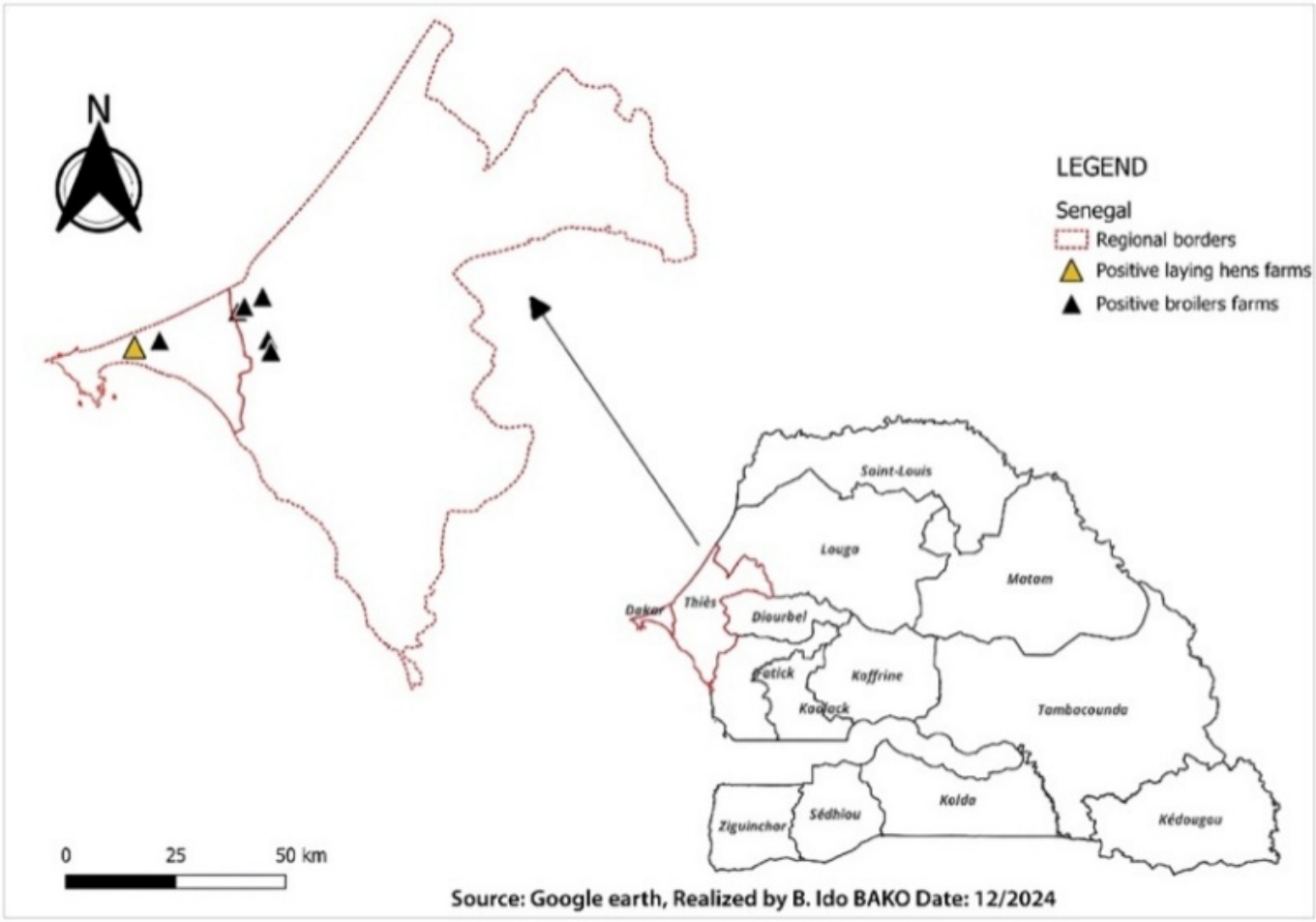

Figure 2. Maps of the study area showing poultry farms infected by H9N2 virus in Dakar and Thiès regions in 2024.

Genetic Characterization and Phylogeny of the Senegalese H9N2 Virus Isolate

The eight HA gene sequences obtained in this study showed high similarity, with greater than 99.6% nucleotide identity, indicating a genetically homogeneous population of circulating H9N2 viruses. One representative strain, designated A/chicken/Senegal51-2/24, was selected for further analysis. This strain was analysed through partial phylogenetic reconstruction of the partial hemagglutinin gene (segment 4).

The resulting phylogenetic tree (Figure 3) revealed that the Senegalese farm strain belongs to the G1-like lineage and grouped most closely with the Moroccan strain A/chicken/Morocco/Ch16/2016, forming a separate subclade. Although this grouping is supported by a low bootstrap value (19), the short branch length and high nucleotide identity (98.9%) between these two strains suggest a close genetic relationship, likely reflecting recent common ancestry or regional circulation between West and North African strains.

In contrast, the Senegalese strain did not cluster tightly with the Algerian strains from 2017, which instead formed a well-supported and distinct monophyletic group, with a bootstrap value of 91. However, the genetic identity values between the Senegalese and Algerian strains (97.1-97.4%) indicate a relatively close evolutionary relationship. The high genetic similarity, as shown by these identity values and the phylogenetic analysis, highlights the close relationship between the strains (Houadfi et al., 2016; Naguib et al., 2017; Fusade-Boyer et al., 2021). No zoonotic-related molecular markers were identified in the Senegalese strain, and the sequences appeared genetically homogeneous.

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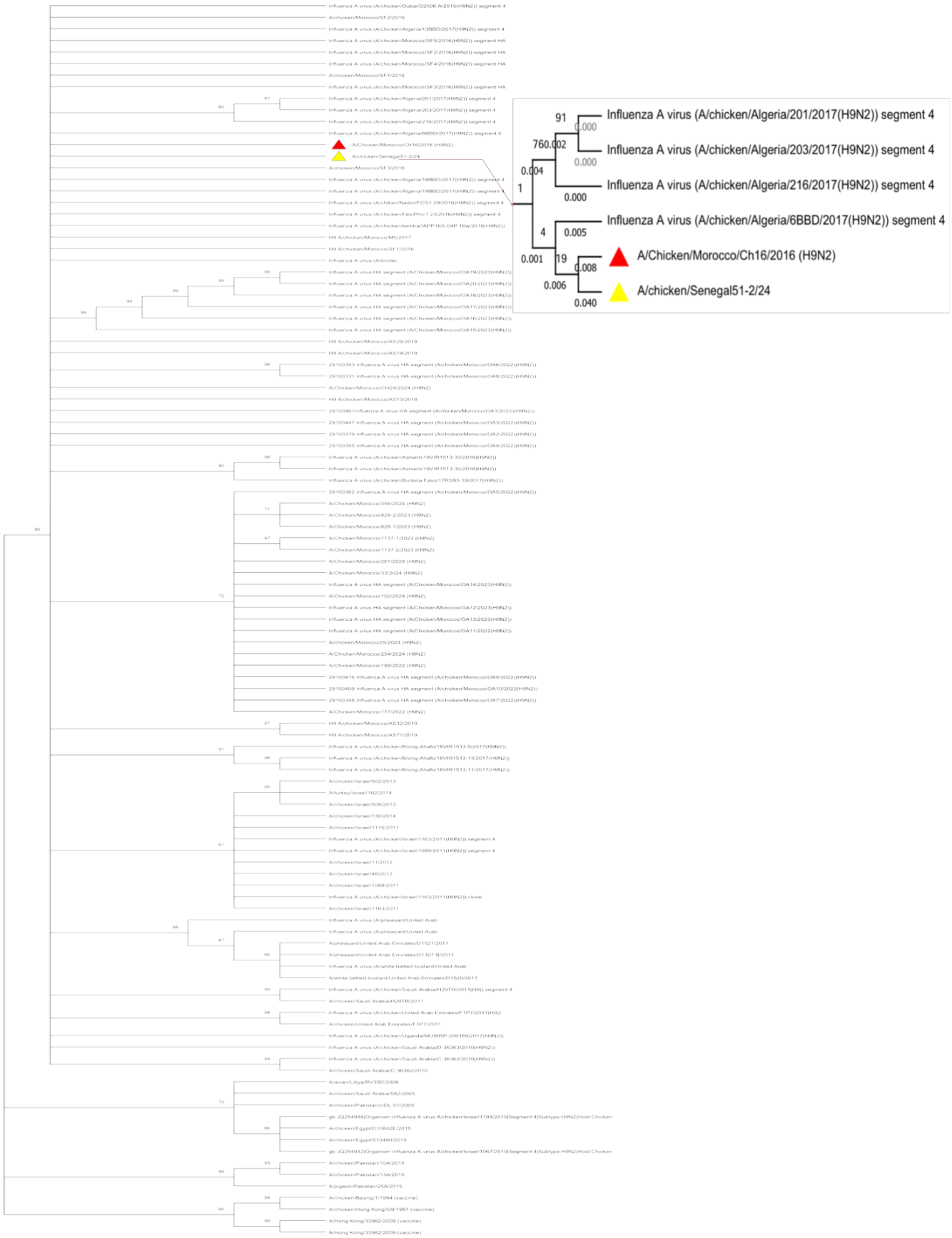

Figure 3. Phylogenetic Tree of Influenza H9N2 Virus Isolates from Dakar et Thiès, Senegal (2024), and Their Evolutionary Relationships with Algerian and Moroccan Strains.

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Discussion

This study aimed to assess the prevalence and genetic character of the circulating low pathogenic avian influenza (LPAI) H9N2 virus in broiler and layer poultry farms in the Dakar and Thiès regions of Senegal. Through molecular screening and phylogenetic analysis, the study provides an insight into the epidemiological and evolutionary current situation of the H9N2 virus in Senegalese commercial poultry farming systems.

The prevalence of H9N2 (9.2%) detected in the current study was far lower than the 58.3% reported by in live bird markets in Tilène and Thiaroye in Senegal. This lower detection rate may be attributed to the use of passive surveillance in our investigation, which likely missed asymptomatic or subclinical infections, and to the timing of sample collection, which may not have coincided with peak viral shedding. Furthermore, while FTA cards offer logistical advantages for field sampling, they may yield lower RNA recovery compared to standard viral transport media. Another possible explanation is that H9N2 is either newly introduced or circulates sporadically within poultry farms in the Thiès and Dakar regions, resulting in genuinely low virus prevalence. Importantly, the current study focused on broiler and layer farms, whereas Jallow et al. conducted surveillance on live bird markets that host a more heterogeneous population of birds varying in species, origin, age, and health status. A similar pattern was observed by in northern Benin, where high H9N2 seroprevalence was recorded in guinea fowl (52%) and indigenous chickens (35%). In addition, comparable high prevalence rates have been recorded in other geographical locations, including Mali (14%), Ghana (24.9%), Tunisia (54%), and Morocco (58%) . Collectively, these findings support the notion of endemic circulation of H9N2 avian influenza in North and West Africa.

The H9N2 virus was identified in 19.4% of broiler pooled samples compared to just 2.0% in layers. These findings suggest a higher susceptibility or exposure risk in broiler production systems. This may be attributed to specific features of broiler farming, such as high stocking densities, shorter production cycles, and generally lower levels of biosecurity . Also, it has been discovered that H9N2 is of low infectious dose and high transmissibility, particularly in the case of broilers .

Geographical mapping of the H9N2-positive farms showed that the majority (75%) were located in the Thiès region, suggesting a potential spatial clustering in this area. This pattern aligns with findings reported by Ba et al. (2022), confirming Thiès as a major poultry-producing region in Senegal and underscoring its priority for enhanced surveillance and control measures. The higher number of farms infected in Thiès compared to Dakar is perhaps due to the greater density of poultry and farming activity in the region. Such trends were also reported by Onidje et al. in Benin, where the farms located in high farm density areas, near forests, or where there was mixed-species rearing were associated with high avian influenza prevalence . Although migratory birds are not considered significant reservoirs of G1-lineage H9N2 viruses , their congregation at sites such as the Djoudj Bird Sanctuary may still contribute to viral reassortment or transient introductions of avian influenza viruses, increasing the potential for genetic mixing with poultry strains .

Phylogenetic analysis of the farm strain (A/chicken/Senegal51-2/24), isolated in this research, revealed that it belongs to the G1 lineage, a line reported to be circulating in countries such as Nigeria, Mali, Burkina Faso, Morocco, and Tunisia . This lineage is believed to have originated in Asia and later spread into North Africa before extending to West Africa. The Senegalese farm strain was genetically close to Moroccan strains A/chicken/Morocco/SF1/2016, which are themselves related to strains from Algeria, Israel, Pakistan, and the UAE . Although poultry imports have been banned in Senegal since 2005, hatching eggs mainly from Brazil and Morocco continue to be imported. Given the phylogenetic proximity of the Senegalese and Moroccan strains, it is plausible that transboundary introduction through hatching egg imports may have played a role. However, further data on trade flows and virus surveillance are needed to support this hypothesis. Moreover, the observed similarity to Algerian and Asian strains raises the possibility of multiple introductions into Senegal . Senegalese H9N2 farm strain lacked molecular markers for zoonotic transmission and increased virulence, like certain Malian strains . Both strains belong to the G1 lineage and share a common ancestor with Moroccan viruses since 2015, highlighting the key role of the importation of hatching egg from Morocco in West African circulation of H9N2 .

In contrast, reported that Senegalese market strains cluster with Asian (Chinese) strains, indicating occasional introduction during illegal imports. The low pathogenicity of both Senegalese market strains and Senegalese farm strains supports previous findings that H9N2 rarely results in substantial poultry mortality . However, the Senegalese market strain reported by has the Q226L mutation in the HA gene that enhances human receptor binding and is zoonotic in regard. Mutation was not found within the farm strain, though the correspondence with Moroccan viruses with it necessitates surveillance . While this research confirms only the circulation of H9N2 in broilers and layer hen farms, documented co-circulation of H5, H7, and H9 subtypes in markets, with increased reassortment risks documented in Asia .

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This study included participating poultry farms that had reported suspicious respiratory disease to veterinary clinics. As a result, farms with asymptomatic, subclinical, or mild infections that had not consulted a veterinarian were not sampled, which could underestimate the true incidence of H9N2. In addition, the reliance on passive surveillance using veterinary reporting may introduce selection bias by only including farms with severe clinical signs, which could miss more subtle infections. Moreover, the study was conducted only in the Dakar and Thiès regions, which, despite representing an important percentage of Senegal's poultry production, may not reflect the whole epidemiological status of H9N2 Virus infection at the country level.

Conclusion

The study confirmed the circulation of low pathogenic H9N2 avian influenza virus in Senegalese broiler farms of Dakar and Thiès regions. Broiler farms showed a higher infection rate than layer farms, likely as a result of differences in management and biosecurity practices. The genetic analysis indicated that the strain circulating was of the G1 lineage and was genetically close to Moroccan strains, implicating regional spread linked to poultry trade. Importantly, the strain detected lacked molecular markers of increased virulence or zoonotic potential, representing a currently low risk to either poultry mortality or human health. These findings emphasize that H9N2 is endemic in Senegal's principal poultry-producing regions and underscore the need for increased disease surveillance to prevent potential outbreaks and economic losses.

In light of these findings, surveillance should be expanded to healthy flocks and to other regions of Senegal. Biosecurity, especially in broiler farms, should be increased to reduce viral spread. Ongoing genetic surveillance is also recommended to detect any new strains of increased risk. Cooperation among veterinary authorities, farmers, and researchers will be key to successful control and to safeguarding both poultry and public health.

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Ethical approval

Owner informed consent was obtained prior to sample collection, and all procedures adhered to ethical guidelines ensuring animal welfare and biosecurity.

Conflict of interest

The authors declare no conflict of interest.

Author Contributions

The research concept was developed by M.C.K.; samples collection was done by A.B.I.B., A.K.C., and S.S.S.; laboratory analyses were conducted under the supervision of S.F.; the initial manuscript draft was prepared by E.O. All authors have given their approval for the publication of this article.

Data availability

The data supporting this study are available upon reasonable request from the corresponding author, Mireille Catherine Kadja.

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