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Epidemiology and evolutionary dynamics of H9N2 avian influenza virus in Bangladesh

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ABSTRACT

Low pathogenicity avian influenza (LPAI) H9N2 has been enzootic in Bangladeshi poultry since 2006. H9N2 outbreaks can decrease egg production and growth and pose a risk to human health. The role of avian hosts in the persistence, evolution, and dispersion of H9N2 is poorly understood in Bangladesh. Hence, this study unveils the intricate role of major host species in virus maintenance and evolution and the temporal and seasonal patterns of H9N2 in Bangladesh from 2006 to 2023. Multinomial logistic regression analysis indicated that the circulation of H9N2 in different species and interfaces is significantly influenced by the seasons. Bayesian phylogenetic analysis of H9N2 sequences in Bangladesh revealed two distinct lineages: G1 and Eurasian. The G1 lineage split into two clusters, coexisting until 2019, at which point only one cluster persisted. Bayesian phylodynamic analysis of G1 lineage unveiled frequent bidirectional viral transitions among ducks, chickens, and quails. Chickens might be a pivotal source of H9N2 in Bangladesh, with a higher number of viral transitions from chickens to ducks and quails. Quails appear to acquire most of their viral transitions from chickens rather than ducks, suggesting that quail epizootics are primarily triggered by spillover events from chickens. Our results suggest viral circulation in commercial chickens despite vaccination. The vaccination approach should be revised, assess vaccine efficacy, and extension of vaccination to backyard chickens and quails.

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KEYWORDS Phylodynamics; surveillance; LPAI; AIV; zoonotic; vaccination; poultry

Introduction

Avian influenza viruses (AIV), notably the H9N2 subtype, represent a persistent challenge to global poultry industries, with implications for both animal and human health. Globally, the prevalence of H9N2 has risen steadily, and the virus has become endemic in poultry populations across Asia, the Middle East, and beyond [1–3]. The ability of H9N2 to reassort and exchange genetic material with other influenza subtypes poses a perpetual threat, with the potential for the emergence of novel strains with increased transmissibility and pandemic potential [4–6].

H9N2 was first detected in domestic poultry in Bangladesh in 2006 [7]. While it typically induces mild symptoms in poultry when infected, it is noteworthy for its impact on reproductive aspects, leading to decreased egg laying and hatching [8,9]. H9N2 is now the predominant form in Bangladesh, extending its transmission to various settings such as live bird markets (LBMs) in both urban and peri urban settings [10,11], commercial farms [12,13], and backyard farms [14]. According to the World Health Organization (WHO), there have been 99 confirmed human cases of H9N2 avian influenza globally [15]. In Bangladesh, after the first detection of the virus in humans in 2011, a total of 3 human cases have been reported [16].

Genetic studies have shown that H9N2 influenza viruses are categorized into North American and Eurasian lineages [17]. The North American lineage is exemplified by WI/66-like, while the Eurasian lineage comprises distinct sub lineages such as BJ/94-like, G1-like, Korea-like, and Y439-like [17]. Recent studies examining H9N2 viruses in Bangladesh have revealed that the virus was like G1 clade while emphasizing their distinctiveness from other G1 viruses [18]. Notably, the genetic similarity observed aligns closely with H9N2 viruses isolated from Pakistan and the Middle East, indicating a regional interconnectedness of

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these avian influenza strains [19]. Furthermore, the ongoing evolution of the H9N2 virus in Bangladesh has been substantiated by recent research [20]. A concerning aspect of this evolutionary process is the virus's expanding host range within Bangladesh. Beyond its initial identification in chickens, H9N2 has transmitted into multiple avian species, including ducks, quail, pigeons, and turkeys which have had a profound impact on the poultry industry, posing challenges to the economy [8,21–23]. Furthermore, a human influenza surveillance in Dhaka detected H9N2 infection in an individual who had evidence of exposure to a sick quail emphasizing the potential public health impact of H9N2 transmission among various avian species [24]. Since 2020, the Bangladesh government has initiated the vaccination of commercial chickens against H9N2 [25]. However, H9N2 has also been identified in various chicken strains within commercial farms, despite the implementation of vaccination programs [13]. A critical question remains regarding whether the circulating strains in Bangladesh differ from the vaccine strain. Thorough investigations and studies are essential to assess the effectiveness of the vaccination strategy, monitor any genetic variations in the circulating H9N2 strains, and provide insights for future control measures. The continuous evolution of H9N2 is characterized by a gradual accumulation of mutations, resulting in the increasing differentiation of BD H9N2 viruses from other lineages circulating in neighboring countries [20]. The most alarming aspect is that H9N2 AIV can serve as a donor of internal genes for the emergence of zoonotic influenza viruses that have the potential to cause a pandemic, such as H7N9, H10N8, H5N1, and H5N6 [9,26]. Hence, H9N2 presents a significant risk to both the poultry sector and public health.

H9N2 virus also has demonstrated varying degrees of pathogenicity across different species, including chickens, quail, ducks, and humans. In poultry, H9N2 infections can lead to significant economic losses, particularly in chickens and quail, which may exhibit clinical symptoms ranging from mild respiratory distress to severe systemic illness [27]. In contrast, ducks often show minimal clinical signs [27], contributing to the complexity of H9N2 epidemiology and its transmission dynamics. Understanding the pathogenicity of H9N2 across these species is crucial for effective surveillance and control measures. To formulate effective mitigation strategies, a comprehensive understanding of how H9N2 spreads within its host community and the specific roles played by different host species (i.e. amplifying, maintenance, dead-end) is imperative. Phylodynamic, a field of study that investigates how epidemiological, immunological, and evolutionary processes influence viral genetic diversity, proves invaluable in this attempt [28]. This approach enables the reconstruction of viral dispersal patterns and evolutionary processes, even when genomic data is sampled relatively sparsely from an infected population [29]. AIVs, including H9N2, exhibit exceptionally short generation times, high evolutionary rates, and large population sizes [30]. Consequently, genetic substitutions in viral genomes occur on similar timescales as transmission events between hosts. Through time-scaled phylogenies, which contain a "molecular footprint" of viral spread, it becomes possible to gather insights into the intricate interplay between the virus and its host community [31]. Notably, there is a scarcity of literature on host dynamics in Bangladesh, particularly in identifying which host serves as an amplifier for the virus. Bayesian phylogenetic analysis and discrete analysis have been underutilized in exploring host roles in the context of H9N2 in Bangladesh. Hence, we investigated and characterized the roles of hosts in the epidemiology and viral evolution of the H9N2 virus in Bangladesh.

Materials and methods

Epidemiological data acquisition and processing

Firstly, we amplified 16 H9N2 sequences from backyard chickens in Bangladesh, which were deposited to GenBank (accession IDs OR751829-OR751844) as part of our ongoing research on backyard poultry farming biosecurity practices conducted in 2021. We selected households that kept backyard chickens and sampled 2–4 birds from each, depending on flock size. Oropharyngeal and cloacal swabs were collected from each bird. Initial screening for AIV was performed using rRT-PCR targeting the matrix (M) gene, following CDC and Spackman protocols [32,33]. Positive samples were further tested for H5, H7, and H9 subtypes using subtype-specific primers and probes using rRT-PCR assay [33]. We used the Nanopore MinION-based influenza sequencing approach to whole-genome sequencing (WGS) of H9 positive samples directly from swab samples following our in-house culture-independent, high-throughput native barcode amplicon sequencing methods and library preparations as previously described [34,35].

Secondly, we used genomic sequences of H9N2 virus originating from Bangladesh, along with their associated epidemiological metadata such as collection dates, host species, and geographical locations. We conducted a systematic search in the Global Initiative on Sharing All Influenza Data (GISAID) [36] and GenBank databases [37] using key terms such as "H9N2," "Bangladesh," "avian influenza," and "LPAI". We focused on sequences originating from

Bangladesh between 2006 and 2023. The data we acquired included both those linked to specific outbreaks and those collected from non-outbreak situations. The sequences data were generated from multiple sample types such as oropharyngeal swabs, cloacal swabs and environmental samples. The environmental samples were from LBM and farm environment sites like pooled samples from floor, slaughter area, waste bin, poultry cage, water, fecal material on or underneath the bird cage, poultry offal. We cross-referenced this information with the National Center for Biotechnology Information (NCBI) influenza virus database [38] to ensure completeness and accuracy.

To ensure data quality and relevance, we applied a series of filtering criteria. Sequences of laboratory-derived viruses and those lacking essential metadata were excluded. Specifically, we excluded sequences that did not contain complete hemagglutinin (HA) or neuraminidase (NA) genes, or those with ambiguous or incomplete metadata (such as host species or collection locations). This process ensured that our final dataset contained only samples with robust and complete information relevant to our study objectives. To further enhance the dataset, we manually reviewed and supplemented the missing metadata from published articles, where possible. This approach not only enriched our dataset but also increased the accuracy of our analysis. This curation resulted in a final dataset containing 568 samples that met our inclusion criteria for epidemiological and evolutionary analysis. This methodology ensured that our analytical dataset was not only comprehensive but also carefully curated, enabling us to draw meaningful insights into the dynamics and distribution of H9N2 avian influenza in Bangladesh.

Statistical analysis

We used R studio version 2022.02.2 [39] for the statistical analysis. Using the ggplot2 package, we presented the temporal trend of H9N2 over the 18 years and proportion of H9N2 across host and interface. We used seasonal decomposition to observe any seasonal component in H9N2 cases. For our analysis, we defined November-December and January-March as the winter season, April-June as summer, and July-October as the monsoon season. We aimed to investigate how host type, farming system and LBM factors influenced the seasonal distribution of the virus. To assess the effects of host type and interface on the seasonal dynamics of H9N2, we employed multinomial logistic regression using the R package nnet.

Multinomial logistic regression is particularly suitable in this context as it allows us to model a categorical outcome variable with multiple levels – in our case, the different seasons in which H9N2 was detected. This analytical approach enables us to estimate the probabilities of each seasonal outcome based on the independent variables of host type and interface, thereby providing insights into how these factors contribute to the observed seasonal patterns of H9N2 circulation. Odds ratios for the model estimates were plotted using the package sjPlot.

Phylogenetic analysis

We constructed two phylogenetic trees based on the HA gene. The first tree presented the HA gene of H9N2 of all hosts including environmental sequences identified in Bangladesh since 2006. The second tree focused on host dynamics and sequences retrieved from poultry species chicken, duck, and quail. We used 400 sequences that were more than 1500 in length and included 16 primary H9N2 sequences from backyard chicken resulting in a comprehensive compilation of 416 sequences for the first tree. Using BEAST v.1.10 [40], data were analyzed with an uncorrelated lognormal relaxed molecular clock and the HKY + G [41], and a constant size tree prior. The tree was run with 10 million steps sampled every 1,000 steps. The maximum clade credibility (MCC) tree was generated using Tree Annotator within the BEAST software and visualized using FigTree 1.4.2. (http://tree.bio.ed.ac.uk/software/figtree/).

Host dynamics via discrete trait analysis

For the second tree, we excluded environmental sequences those species host mentioned and particularly focused on within kwon host species chickens, ducks, and quails, yielding a final dataset of 314 sequences. BEAST v.1.10 was used to analyze data [40] using an uncorrelated lognormal relaxed molecular clock, the HKY + G, and a constant size tree prior. We analyzed chickens, ducks, and quails' sequences to determine ancestral host states and estimate asymmetric viral exchange across host species. The asymmetric substitution model was used for discrete trait analysis, while Bayesian Stochastic Search Variable Selection (BSSVS) was used to infer social networks [42]. The amount and pattern of viral migration across hosts were estimated using SpreaD3's Bayes Factor analysis [43]. A transition was considered significant when the posterior probability was greater than 0.5, and, in accordance with Jeffreys [44], viral transition rates were considered statistically supported when the Bayes Factor (BF) was greater than 3.0 (arrows with light orange borders), strong support when BF > 10 (arrows with red borders), and decisive support when BF >100 (arrows with black borders). The migratory events across hosts were determined by tracking the

changes between states along evolutionary branches (Markov Jumps) [45].

Results

Temporal and seasonal trend of H9N2 cases in Bangladesh

Over the past 17 years, H9N2 cases were consistently reported annually in Bangladesh, with the exception of 2008 (Figure 1). In 2016, the highest number of H9N2 cases was reported, with 171 cases, accounting for 30% of the total cases reported during this period. The second-highest number of cases occurred in 2017, with 74 reported cases, representing 13% of the total cases. It is noteworthy that in 2016 and 2017, H9N2 cases were reported every month, except for March 2016. In 2018, 48 cases were reported. Subsequently, the number of cases decreased to 20 in 2019 and 30 in 2020. However, there was an increase in cases in 2021, with 43 reported cases.

Additionally, we found that over the years 2006–2022, more than half of the reported cases of H9N2 in Bangladesh came from chicken samples (55.8%), making chickens the most common host. The second-highest source, accounting for 28.3% of cases, was environmental samples. If we look closely at the timeline, we notice some interesting trends. Initially, in 2006 and 2007, H9N2 was only found in chickens (Figure 1). But by 2009, it started appearing in environmental samples and in quail. This continued, with cases consistently showing up in both chickens and environmental samples each year. Quails also remained a frequent host.

Seasonal patterns of H9N2 in Bangladesh

The analysis of H9N2 cases in Bangladesh from 2006 to 2022 revealed distinct seasonal patterns. Notably, 50% of these cases were detected during the winter season, with an additional 37% occurring in the monsoon season. In contrast, a mere 13% of the cases were reported during the summer season. This rise in H9N2 cases during the monsoon and winter seasons, suggesting a pronounced seasonal trend in the data. For further visualization we used time series decomposition for the H9N2 cases, and we can see that a seasonal component is present in the time series (Supp Figure 1).

Significant associations between seasonal variations and the presence of H9N2 at the interface were observed in univariable analysis (Figure 2). In commercial farms ($N = 30$), the winter season emerged as the predominant period for H9N2 detection, constituting a significant portion of cases at 70%. Conversely, in backyard farms ($N = 26$), a contrast was evident, with no reported cases during the monsoon season, but a substantial 58.6% of detection during the summer months. LBMs ($N = 512$) also exhibited seasonal variation, with H9N2 cases primarily concentrated during the monsoon season (47.9%), followed closely by the winter season (40%).

Our univariable analysis further underscored the existence of a significant association between the season and the host species (Figure 2). In the case of chickens ($N = 313$), the virus was consistently detected throughout all seasons, with the highest incidence during winter (42.5%), followed by summer (23.6%) and monsoon (33.9%). Conversely, ducks ($N = 34$) displayed a distinct pattern, with H9N2 virus detection most prevalent during winter (61.3%), followed

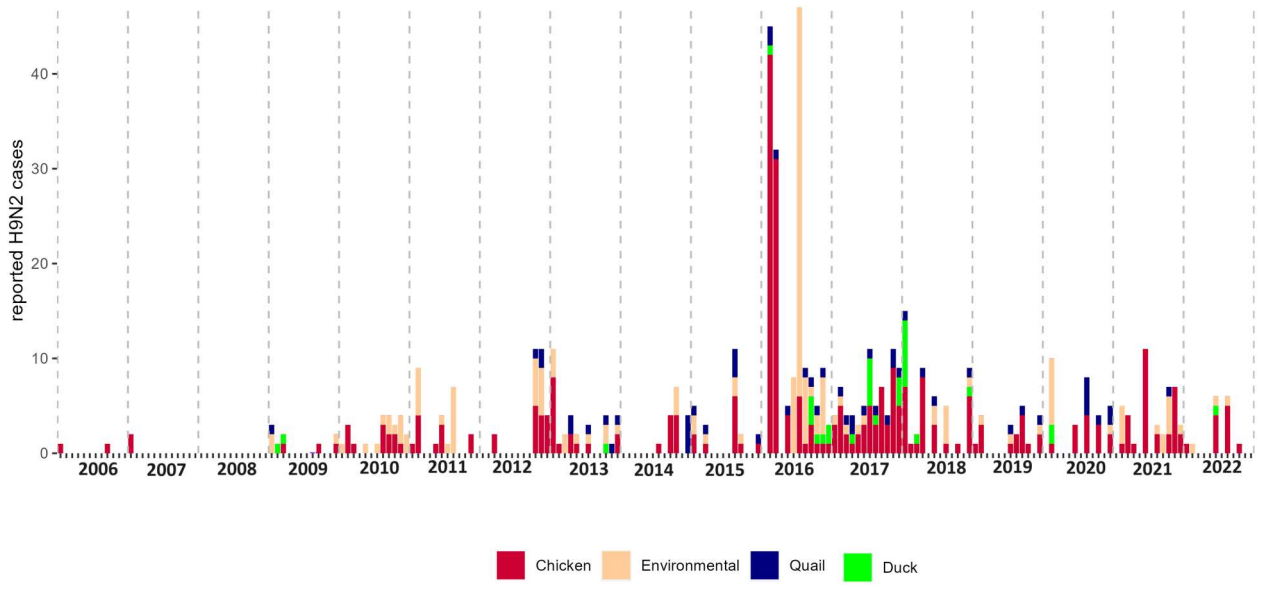

Figure 1. Temporal Trend of H9N2 Cases in Bangladesh (2006–2022) by Species. Each column represents a month, with dotted lines denoting year transitions. The color-coded bars within each month reflect the number of cases reported from different species.

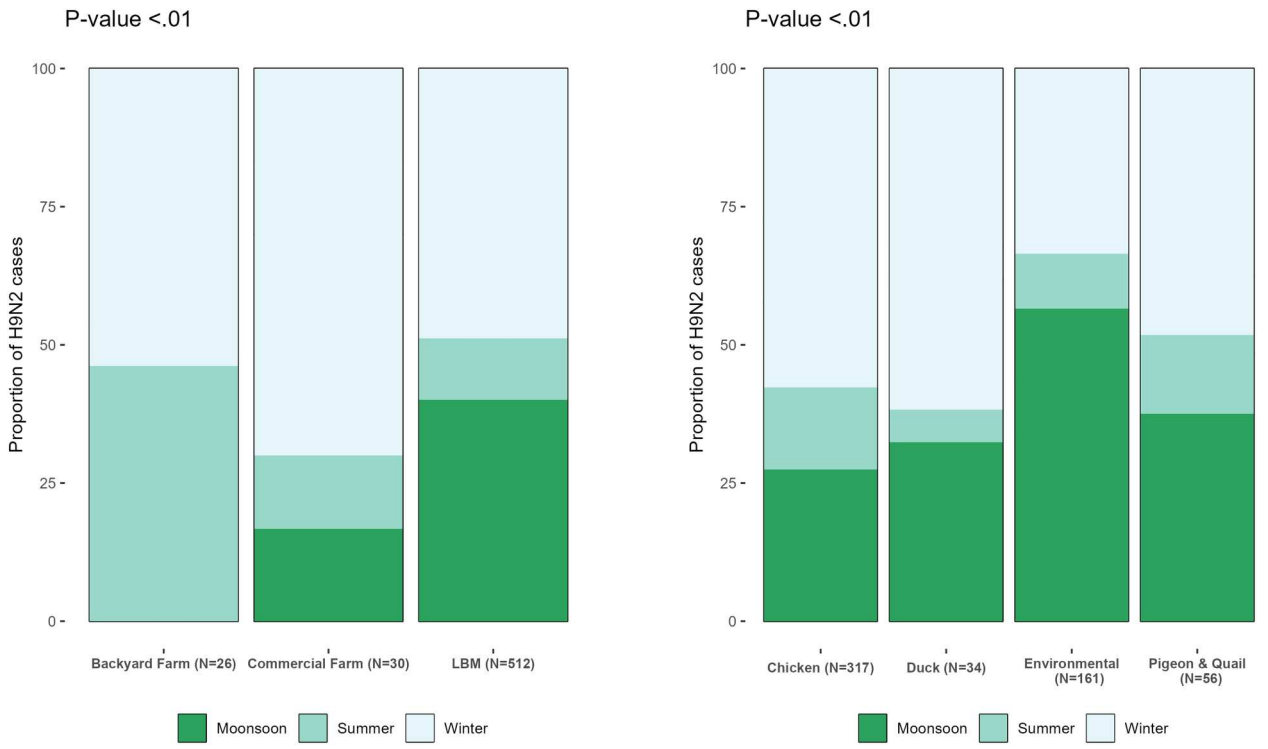

Figure 2. Seasonal patterns of H9N2 cases for species and interfaces. The *P*-value is obtained from Chi-squared test.

by the monsoon season (35.5%), while very few cases were reported during the summer. Environmental samples ($N = 161$) also reflected seasonal variation, with the majority of cases detected during the monsoon season (56.3%), followed by the winter season (35.6%). Similar trends were observed in pigeon and quail populations ($N = 56$), with minimal detections during summer and higher in winter (45.3%) and monsoon (39.6%).

From the multinomial logistic model, both species and interfaces were found to be significant (Table 1, Figure 3). H9N2 detection in duck samples showed a reduced likelihood during summer (Relative Risk Ration (RRR): 0.1) compared to winter. On the other hand, the detection was almost 2 times higher in monsoon than winter in environmental samples. Furthermore, the risk of H9N2 detection in backyard farm samples was nearly 8 times greater in summer than in winter. Conversely, in LBMs the risk of H9N2 detection was approximately three times higher during the monsoon season than in winter.

Phylogenetic analysis and epizootiology of H9N2 across different hosts in Bangladesh

Results from the Bayesian phylogenetic analysis of all H9N2 sequences identified in Bangladesh indicate the presence of two distinct groups: the G1 lineage and the Eurasian lineage of H9N2 viruses (Figure 4). Most sequences from Bangladesh fall within the G1-like lineage. Through time-structured phylogenetic analysis, it has been revealed that this particular clade entered Bangladesh around the end of 2005. The G1-like lineage has since become established in the country. Viruses from the G1 lineage have been found in various hosts, including the environment, waterfowl, chickens, and quail. The G1 lineage has split into two separate clusters. Cluster 1 began circulating in Bangladesh around July 2009, and cluster 2 emerged in March 2010. Both clusters coexisted in Bangladesh until 2019 However, from 2020 onwards, only cluster 1 has been observed in circulation within the country. By the end of 2018, cluster 1 also gave rise to a new subgroup. It reveals that viruses belonging to the G1 lineage in Bangladesh exhibit a continuous process of evolution. In 2016, three sequences originating from ducks, along with one sequence from the environment in 2009, which exhibited resemblances to viruses belonging to the Eurasian lineage. These four viruses were shown to be genetically related to viruses from wild birds of Eurasian origin in Australia (OL370160, OL370153), Iran (FN600116), South Korea (OK342157) and Ukraine (MW183250).

Over time, the G1 lineage of H9N2 viruses has established endemicity in Bangladesh (Figure 5). This lineage comprises as many as fifteen distinct genetic subgroups (R1-R15), identified based on Bayesian phylogenetic tree analysis with a high posterior probability (>99%). Initially, within the H9N2 G1 lineage, chickens were the only host observed in subgroups R1-R7 (Figure 5). However, in subgroup R8 and subsequent subgroups, a notable shift

Table 1. Estimates with standard error and *p*-value of multinomial logistic model relating the variation of H9N2 in species and interface with season in Bangladesh.

		Summer					Monsoon				
Variable	Categories	Estimate	RRR*	std. error	95% CI	*p* value	estimate	RRR*	std. error	95% CI	*p* value
Species	Environmental	0.00	1.00	0.49	0.38–2.61	0.99	0.80	2.22	0.34	1.14–4.34	**0.02**
[Quail]	Chicken	−0.35	0.71	0.44	0.30–1.67	0.44	−0.39	0.68	0.32	0.29–1.72	0.23
	Duck	−2.32	0.10	0.95	0.02–0.63	**0.01**	0.07	1.08	0.50	0.01–0.71	0.88
Interface	Backyard Farm	2.12	8.35	0.73	1.92–38.84	**0.00**	−9.74	0.00	69.79	0.00–35.0	0.89
[Commercial farm]	LBM	0.11	1.11	0.57	0.36–3.41	0.85	1.02	2.77	0.52	0.37–3.35	0.05

Note: The dependent variable in our model was the season, while the independent variables were host type and interface. The category inside the parentheses in the first column is the baseline category for the corresponding categorical variable. The estimates and results presented in this table are compared with the winter season. *RRR: Relative Risk ratio.

occurred, with the frequent observation of virus circulation in quails and ducks alongside chickens (Figure 5). Subgroup R13-R15 also displays a multi-host pattern, with chickens being predominant, but all three avian species chickens, ducks, and quails were observed.

To elucidate the role of different hosts in the epidemiology of the G1 lineage of H9N2 viruses, ancestral state reconstruction was employed to identify viral transitions, or Markov jumps, between key host groups, including chickens, ducks, and quails (Figure 6). Chicken emerged as net donors, continuously engaging in more viral export events than imports. Ducks, on the other hand, took on the role of net receivers (Figure 6). The transition rate data indicated that the most significant viral flow was from chickens to quail, with a transition rate of 14.39 (Bayes factor > 100), while the second-highest transition rate was from chickens to ducks (transition rate 11.21, Bayes factor > 100). In addition to viral imports from chicken, transitions of viruses from quails back to ducks were also observed (transition rate 2.72, Bayes factor > 100) (Figure 6). It is noteworthy that chickens exhibited the longest Markov reward time, indicating that when viruses move into chickens, it takes a considerably longer time for them to transition to another host (Table 2).

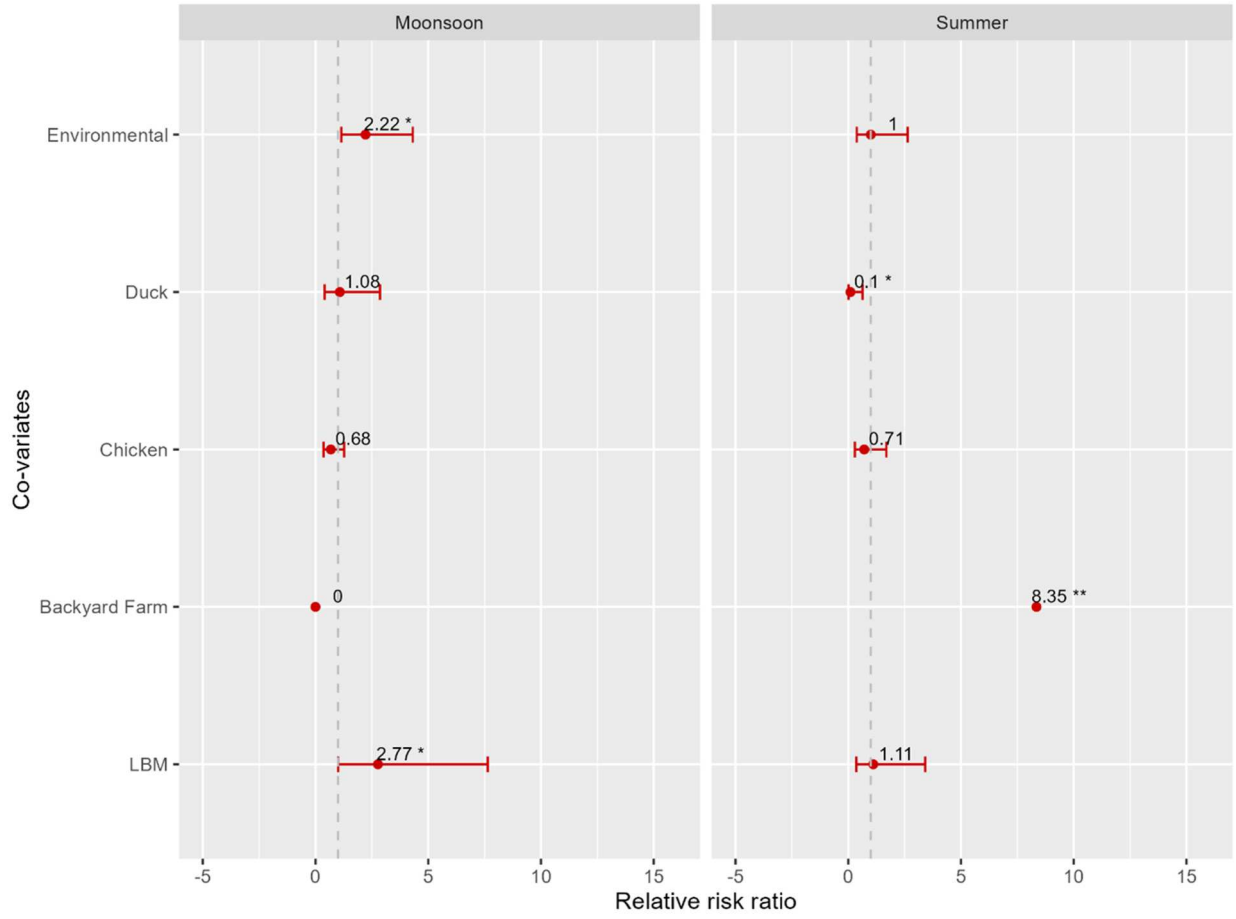

Figure 3. Odds ratio with 95% confidence interval of the variables of the multinomial logistic regression model relating the variation of H9N2 in species and interface with season in Bangladesh (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

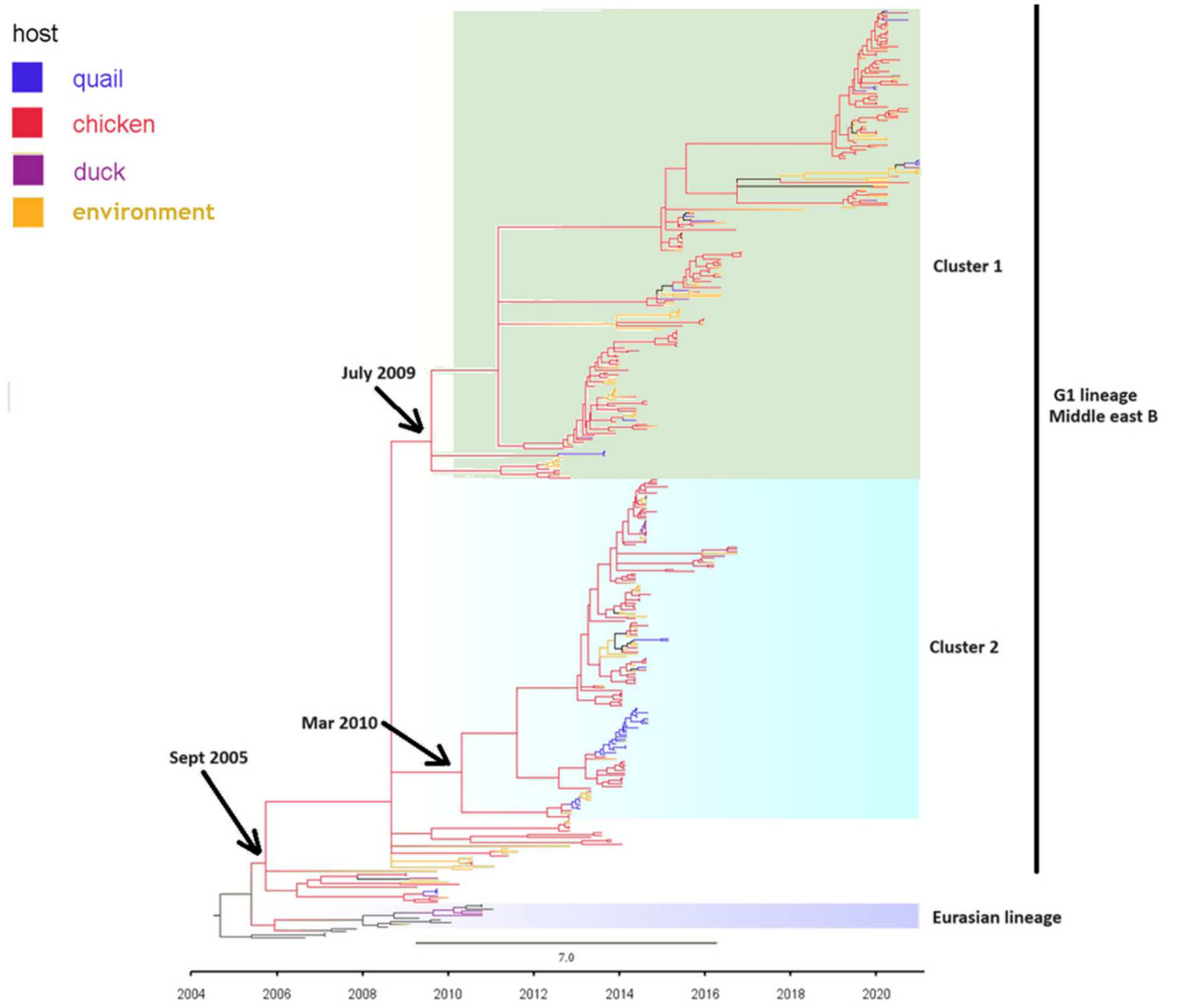

Figure 4. Bayesian Phylogenetic tree of the HA gene of H9N2 in Bangladesh from 2006–2023, depicting the various clades. While the two large grouping at the top of the tree (in blue and yellow) indicates the G1 lineage and the small group (in purple) indicates Eurasian lineage. The scale bar indicates the number of substitutions per site. Colored boxes indicate both HA clade and HA-NA subtype combination. Branches are coloured according to host type.

Discussion

In our extensive 18-year study of H9N2 viruses in Bangladesh, we uncovered a notable seasonal pattern in H9N2 detection. Additionally, by analyzing the evolutionary host dynamics, we traced the trajectory of enzootic H9N2, unraveling the intricate relationships between the virus and its avian hosts over an extended period. Our study revealed that the majority of H9N2 cases in Bangladesh had been detected in chickens, with a significant percentage reported in environmental samples. The widespread detection of H9N2 in the environment can be facilitated by weak biosecurity procedures on poultry farms and LBMs, which allow for viral transmission via contaminated equipment, insufficient waste management, and cramped housing situations [47,48]. Furthermore, poor litter and manure management practices can lead to ongoing environmental infection. Addressing these biosecurity practices is critical to reducing the environmental reservoir of H9N2 and limiting its continued circulation inside poultry facilities.

Our study also found that the detection of H9N2 was higher in winter with half of the cases being detected in this season. Previous studies conducted in Bangladesh and neighboring countries also found that in winter season the risk of H9N2 transmission increases [49–51]. Cold temperatures and low humidity in winter create an environment conducive to the stability and transmission of the virus [52,53]. The combination of cold stress, poor ventilation, and elevated ammonia levels in poultry houses during the winter season can significantly contribute to the increased detection of H9N2 [54]. Furthermore, the potential weakening of poultry immune responses in response to winter stressors enhances the susceptibility of birds to H9N2 infections during colder months [23]. We acknowledge that the lack of detailed information on the number of samples tested across seasons is a limitation of our study. Variations in sampling

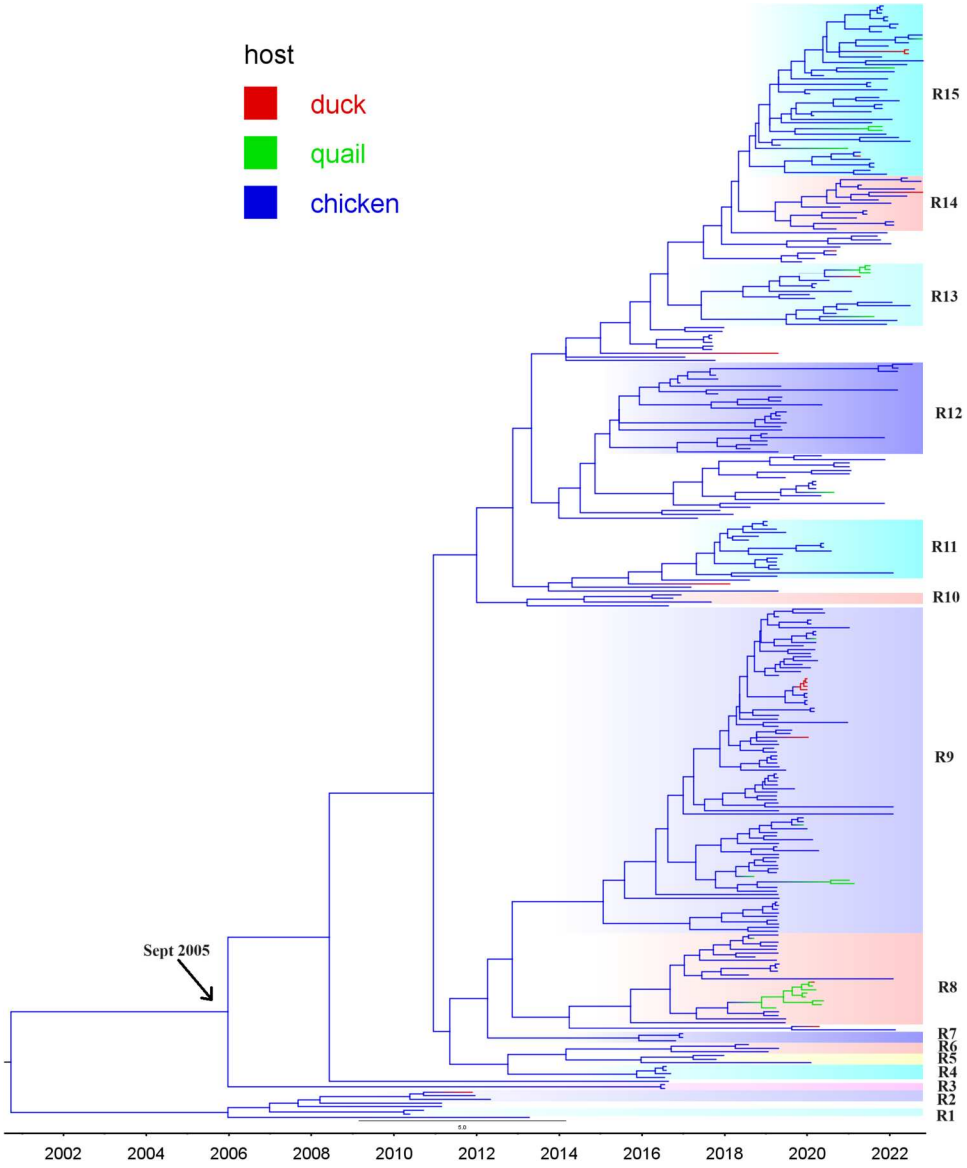

Figure 5. Time-scaled phylogenetic tree of HA sequences of H9N2 in Bangladesh. Branches are colored according to host type and the thickness of branches indicates posterior probabilities of the ancestral host type. Genetic subgroups are identified by differently colored boxes and marked R1-R11.

efforts could potentially distort the seasonal trends observed in H9N2 cases, with higher detection rates in certain seasons reflecting differences in surveillance intensity rather than true seasonal variation. Despite this limitation, our findings are consistent with previous longitudinal studies on H9N2 in other regions, reinforcing the reliability of our observations. Future studies with more standardized sampling across seasons would help reduce sampling bias and provide more robust insights into the seasonality of H9N2 in Bangladesh.

While first clinical case of H9N2 was reported in Bangladesh in 2006 [7], the time structured Bayesian phylogenetic analysis revealed that, introduction of G1 lineage in Bangladesh was around September 2005, consistent with the study conducted by [20]. Introductions of this virus in India (2001–2002) [55] suggests a regional dissemination pattern, possibly facilitated by interconnected poultry trade or migratory bird routes. The subsequent divergence of the G1 lineage into two major clusters, circulating until 2019, mirrors findings observed in neighboring countries like India [56], Egypt [57], and Pakistan [58]. The cluster 1 and 2 are localized clustered predominately and less frequently spatially clustered over two major cities like Dhaka and Chattogram [20]. One of these two clusters stopped circulating in 2020 which matches when Bangladesh started vaccinating against H9N2 viruses [25]. This connection hints that vaccination might have played a role in reducing the presence of that particular viral group. On the other hand, three sequences from duck in 2016 and one sequence from environment in 2009 clustering with viruses originating from Australia, Iran, South Korea, and Ukraine in Eurasian lineage (Korean like) underscores the

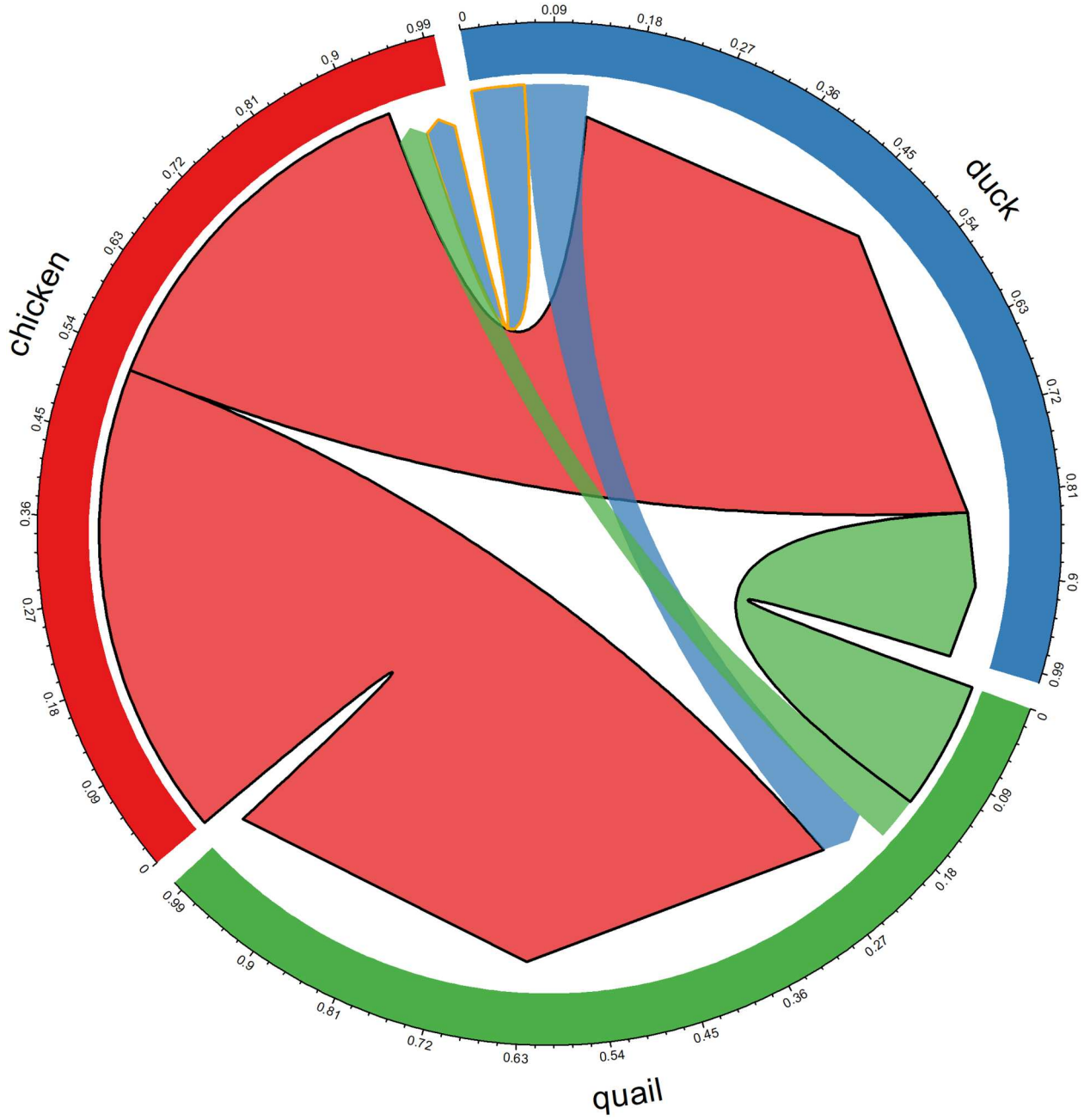

Figure 6. Circos plot of Markov jump counts (reflected by arrow width) illustrating the viral transitions chicken, duck and quail. The direction of arrows indicates the direction of the Markov jumps. Following Jeffreys [46], viral transition rates were considered statistically supported when Bayes Factor >3.0 (arrows with orange borders), strong support when BF > 10 (arrows with red borders) and decisive support when BF >100 (arrows with black borders).

interconnectedness of avian influenza viruses through migratory bird pathways [59]. Previous studies in Bangladesh highlighted the critical role of the wetland ecosystem in the transmission of LPAI viruses between domestic ducks and wild migratory birds [60]. They found an overlap of the free-range duck farming season with the presence of overwintering migratory waterfowl which results

Table 2. Mean reward, number of Markov jump and statistical support value between chicken, duck and quail populations in Bangladesh during the 2006–2023 for H9N2.

Transition from	Mean Markov Reward	Transition to	Mean Markov jumps	Bayes factor	Posterior probability
Chicken	2260	Duck	11.22	> 100	1
		Quail	14.40	> 100	0.99
Duck	40	Chicken	1.12	0.80	0.39
		Quail	0.94	7.62	0.86
Quail	59.37	Chicken	0.92	0.79	0.39
		Duck	2.72	112.83	0.99

in shared habitats and create opportunities for virus exchange.

Bayesian discrete trait analysis revealed that the H9N2 virus exhibited a higher transition rate from chickens to both ducks and quail. This underscores the pivotal role of chickens in shaping the viral dynamics in the region. While domestic ducks are thought to have introduced the H9N2 virus to Asia [61], previous research has shown that chicken may have been the means of introduction via cross-border trade or illegal trading from Pakistan, most likely via India, to Bangladesh, [18] and our analysis indicates that chickens are serving as the amplifying host. Notably, the Markov reward time was found to be higher in chickens than in ducks. This finding resonates with earlier research that highlighted an extended duration of H9N2 virus presence in Phasianidae (such as chickens, quails and pheasants) compared to Anatidae (such as ducks, geese, and swans) and Neoaves (such as pigeons, sparrows and falcons) [59]. Prior studies conducted in Bangladesh also implicated that H9N2 was more frequently detected in chickens across both farm [62] and LBM [63] settings compared to ducks. H9N2 viruses can replicate efficiently in multiple organs of chickens, and this virus can generally shed in high titers from the respiratory tract along with intestinal tract [64]. Therefore, H9N2 viruses could be transmitted efficiently among contact chickens via the respiratory tract and intestinal tract. While H9N2 infections typically manifest with mild or asymptomatic symptoms in chickens, the associated production losses pose a significant challenge for the poultry industry [65,66]. Beyond the economic implications, H9N2 works as a potential mixing vessel, facilitating the reassortment of AIVs and contributing to the emergence of highly pathogenic AIVs [67,68] and amplifies the risk of novel and more virulent influenza strains, requiring heightened surveillance and control measures to prevent the potential spread of these viruses.

Furthermore, our analysis revealed a substantial viral transmission from quail to ducks, indicating a complex interplay among different avian hosts. Previous studies have also shown that H9N2 viruses are widespread across Asia and the Middle East, particularly in chickens and quail [69]. Earlier studies in China have also documented the significant role of quail in the evolutionary dynamics of influenza viruses, serving as intermediate hosts wherein avian influenza viruses undergo amplification and subsequent transmission to other animal species [70]. On the other hand, in quail isolate, significant genetic variability was identified in the NA gene in Egypt [71]. This genetic diversity has the potential to influence the virus's pathogenicity in poultry and its transmissibility to humans, posing implications for both animal and public health.

Our findings challenges the conventional focus of vaccination on commercial chickens [25]. Contrary to current vaccination practices limited to commercial chickens, our data reveal a substantial proportion of H9N2 viruses in quails. Expanding the scope of vaccination programs to encompass not only commercial chickens but also backyard chickens and quails emerges as a crucial strategy. Our study highlights the need for a holistic approach to vaccination, recognizing the diverse avian species involved in the maintenance and spread of H9N2. However, acknowledging the limitations of vaccination as the sole solution, we should emphasize the imperative to fortify biosecurity measures. Prior research has revealed instances of the silent spread of H9N2 through an antigenic drift and evolving into different antigenic groups despite vaccination efforts in Korea [72].

Our investigation also revealed the continuous evolution of the virus in Bangladesh. The emergence of multiple subgroups within the H9N2 lineage highlights the adaptability and genetic plasticity of the virus over time. Furthermore, the presence of multiple avian influenza strains within the poultry population can create opportunities for genetic reassortment, potentially leading to the emergence of new, more virulent strains that could pose greater risks to human health. The reported human cases in Bangladesh have primarily occurred in individuals with direct or close contact with infected poultry [16]. This emphasizes the crucial link between human infections and the dynamics of the virus within avian populations. The implications of these findings underscore the need for enhanced surveillance of both avian populations and potential human cases.

The implementation of stringent biosecurity practices, encompassing proper farm management, restricted movement, and hygiene protocols, is vital to complement vaccination strategies and mitigate the risk of H9N2 transmission. In addressing the efficacy of vaccination as a critical measure to contain the spread of H9N2 in Bangladesh, it is important to acknowledge the challenges posed by antigenic drift in the virus. Similar to the situation in China, where H9N2 continues to circulate despite long-term vaccination programs, the rapid mutation of the virus has led to the emergence of distinct antigenic groups that evade current vaccine-induced immunity [73]. Studies have demonstrated that while vaccines can reduce shedding of earlier antigenic groups, they fail to provide sufficient protection against more recent strains [72,73]. This highlights a significant issue where the speed of virus mutation outpaces the development and update of vaccines. Given that antigenic variation and incomplete vaccine coverage can contribute to the persistence of H9N2, it is essential that we assess the efficacy of the current vaccination

practices in Bangladesh. Regular updates of vaccine seed strains and close monitoring of circulating viral variants will be necessary to ensure optimal protection. Therefore, a multi-faceted approach, combining vaccination with improved biosecurity measures and enhanced viral surveillance, is crucial for controlling H9N2 in Bangladesh. Given the potential for genetic reassortment and the emergence of novel strains, ongoing awareness is vital to inform timely interventions and prevent the escalation of H9N2 into more severe forms. The limitations of our study include the potential contribution of under-sampled or unsampled populations to H9N2 spread, despite efforts to minimize bias by including all available sequences. It should also be noted that, the high incidence of H9N2 outbreaks in chickens [27], supports our findings that chickens play a pivotal role in the introduction and dissemination of H9N2 in Bangladesh.

Our phylogenetic analysis indicates host adaptation of H9N2, particularly in chickens, ducks, and quails, but without in vivo or in vitro replication studies, it remains unclear whether chickens facilitate better viral replication or if their high infection rates are due to increased exposure in poultry farms. Additionally, our findings suggest that current vaccination strategies may be insufficient, as H9N2 continues circulating despite vaccination. However, direct evidence of vaccine-induced immune evasion remains to be established. Antigenic drift analyses comparing vaccine strains with circulating viruses and serological testing of vaccinated versus unvaccinated birds would clarify whether waning immunity is a factor. Studies from China and Korea have shown that H9N2 evolves antigenically even under long-term vaccination programs, leading to immune escape variants and continued viral circulation [72,73]. Given that antigenic variation and incomplete vaccine coverage can undermine control efforts, future research should assess vaccine efficacy in Bangladesh to guide more adaptive immunization strategies.

A limitation of our study is the inability to distinguish between outbreak-associated and non-outbreak-associated samples due to the nature of H9N2 infections and reporting practices in Bangladesh. H9N2 infections are often asymptomatic or present with mild symptoms, resulting in underreported outbreaks [74]. Furthermore, the lack of a mandatory reporting system and the reluctance of farmers to report H9N2 outbreaks to authorities create gaps in the availability of accurate outbreak data. These challenges made it infeasible to apply re-weighting or sensitivity analyses without introducing additional inaccuracies. Despite this limitation, our approach of treating all samples with equal weight enabled us to analyze a comprehensive dataset representing various hosts, interfaces, and seasons. Future studies should aim for more systematic sampling and detailed data collection to better differentiate outbreak and non-outbreak contexts and incorporate these differences into analyses. Such advancements could further refine the understanding of H9N2 dynamics and strengthen the foundation for targeted interventions.

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Disclosure statement

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Data availability statement

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author.

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