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Research Article

Investigating Pathology and Subtypes (H5 and H9) of Avian Influenza Virus (AIV) Circulating in Ducks, Layers and Broilers

Dilruba Parvin[1], Nazneen Sultana[1], Munmun Pervin[1], Sajeda Sultana[2], Abusaleh Mahfuzul Bari[1], and Mohammad Abu Hadi Noor Ali Khan[1]✉.

[1]Department of Pathology, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

[2]Department of Pathology, Sher-e-Bangla Agricultural University, Sher-E-Bangla Nagar, Agargaon, Dhaka-1207, Bangladesh

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Correspondence
Mohammad Abu Hadi Noor Ali Khan
✉: hadi.khan@bau.edu.bd

ABSTRACT

The avian influenza virus (AIV) is consistently ignited in chickens, ducks and broilers. This study was aimed to investigate the detailed pathology of AIV infected ducks, layer chicken, broilers, and pigeons and subtyping of hemagglutinin (HA) and neuraminidase (NA) genes of AIV to predict types of this virus prevailing in Bangladesh. A total of 32 birds were collected from live bird markets, CDIL (Dhaka), and layer farms. Layer chickens exhibited depression, cyanotic combs and wattles. Necropsy was performed; tissue samples were fixed and stained with hematoxylin and eosin, and examined under microscope. Grossly, the birds showed mild to moderate hemorrhages on tracheal mucosal, congested and consolidated lungs, hemorrhagic pancreas and mild congestion and hemorrhages throughout the body. Histopathology revealed widespread congestion and hemorrhages in the tracheal mucosa, lungs, pancreas, heart, and degenerated hepatic cords. Hemorrhages in pancreas, tracheal mucosa, comb, wattles and or shanks were characteristics. Viral RNA was extracted from tracheal tissues using the PureLink™ Viral RNA Mini Kit, and RT-PCR targeting M, HA, and NA genes of AIV were performed for subtype identification. Out of 32 birds examined, M protein gene specific RT-PCR amplification of 510bp fragment was detected in 18 cases. H5 gene specific RT-PCR found to amplify 499bp fragments in 2 ducks and 4-layer chickens and H9 gene specific (221bp) amplification was found in two broiler chickens. NA subtype gene amplification (1089 bp) revealed 8 birds yielding the expected amplicon. H5 subtype was predominant in ducks and layer chickens, while H9 subtype was detected in broilers. H5, H7, and H9 gene-specific amplification left undetected in 02 pigeons and 08 other birds. AI viruses were prevailing in the birds of live bird markets and it needs more emphasis to routinely sequence and sequence analysis of HA and NA genes of AI viruses to predict future emergence of any pandemic viruses of public health importance and designing its control strategies.

Introduction

The poultry sector in Bangladesh has expanded rapidly over the past two decades and now represents one of the most dynamic components of the country's livestock industry. (Hamid et al., 2017). Poultry keeping also plays a vital socioeconomic role in rural communities, where about 85-90% of households rear small flocks of chickens or ducks for income generation, nutrition and women's empowerment (Rahman et al., 2020; Ushimaru et al., 2025). Consequently, the poultry industry ranging from backyard flocks to intensive broiler and layer farms contributes significantly to employment generation, poverty reduction and overall economic growth. Despite this progress, the sustainability of poultry sector remains constrained by recurrent infectious disease outbreaks. Among these, avian influenza virus (AIV) remains one of the most persistent and economically damaging threats.

Type A avian influenza virus is a member of the genus Alphainfluenzavirus and family Orthomyxoviridae. The virus can be divided into two distinct categories based on their capacity to spread disease as well as specific genetic and biological characteristics: highly pathogenic avian influenza (HPAI) virus and low pathogenic avian influenza (LPAI) virus (Mumu et al., 2021). The three avian influenza subtypes that are now endemic in poultry in Eurasia H5N1, H7N9, and H9N2 are the most likely to infect humans and result in death (Watanabe et al., 2014). The H5 subtype of avian influenza affecting worldwide poultry industry having serious human health and food security implications (Lee et al.,

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2017). Currently, the HPAIV virus (H5N1) is prevalent in chicken populations in several nations, including Bangladesh, China, Indonesia, Vietnam, and Egypt, and it is accidentally transmitted to humans through these same countries (CDC, 2012).

Ducks act as important reservoirs, whereas commercial chickens play a central role in amplifying and maintaining viral transmission within the poultry value chain. Both highly pathogenic avian influenza (HPAI) H5 viruses and low pathogenic avian influenza (LPAI) H9N2 viruses continue to circulate in ducks, layers and broilers, frequently causing respiratory disease, production losses and occasional mortality (Parvin et al., 2020; Islam et al., 2023). Over the course of annual genetic evolution, the viral pathogenicity and transmissibility of H9N2 AI viruses have demonstrated a rising trend to infect the poultry industry. By directly infecting people and giving a portion or the entire cassette of internal genes to create novel human-lethal re-assortants like the H5N1, H7N9, H10N8, and H5N6 viruses, the H9N2 virus has also demonstrated its significance to public health (Gu et al., 2017).

With a history of poultry exposure in live bird markets and commercial and free-range farms, Vietnam, Thailand, Indonesia, Hong Kong, China, and Cambodia have all reported human H5N1 infections, suggesting that both live bird markets and farms may play a role in the spread of AIVs among poultry and from poultry to humans (Hassan et al., 2018).

Throughout the year, Bangladesh has seen recurring outbreaks of the low-pathogenic avian influenza subtype H9N2 and the highly pathogenic avian influenza subtype H5N1 in the poultry industry and birds of live bird markets (Bari et al., 2009; Barman et al., 2019; Parvin et al., 2022; Rahman et al., 2018; Ruba et al., 2015; Sarker et al., 2011). In particular, broiler chickens and ducks are sold in live bird markets which carry the infections throughout the year and affect the cage sailing or free-living birds and there is a hidden threat to generate pandemic AIV. Therefore, the continuous identification of the AI virus as well as the molecular characterization is required. Thus, the present study was designed to identify the pathology and subtypes of AIV from the commercial layer chickens, ducks, broiler birds and selected pigeons.

Materials and method

Sample collection

A total of 32 birds were collected from 7th September, 2021 to 10th February, 2022. Among them 13 samples (11 broilers and 2 ducks) were collected from live bird market, 16 samples (12-layer chickens, 2 ducks, 2 pigeons) from Central Disease Investigation Laboratory (CDIL) and 3 birds from three suspected layer farms of Mymensingh district.

Pathological examination

Postmortem examination was performed on to the collected birds of live bird market or dead and sick birds submitted for necropsy in the Department of Pathology. Gross tissue alterations were noticed during systemic dissection was recorded. For histopathological analyses, representative tissues from the trachea, lungs, liver, kidney, spleen, heart, brain, pancreas, intestine, and gizzard, were collected and fixed in 10% neutral buffered formalin (NBF) and tissues were processed and stained with H&E using standard procedure (Luna, 1968). A part of the samples was also collected aseptically in the Eppendorf tube and preserved at -20^{0}C for viral RNA extraction and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) detection of AIV.

RNA extraction

Viral RNAs were extracted from the trachea of birds using the PureLinK^{TM} Viral RNA Mini Kit (Invitrogen, USA) following manufacturer's instructions. All the extracted RNA were qualitatively and quantitatively tested by using agarose gel electrophoresis and spectrophotometry (A260/A280 ratio). A A260/280 ratio of ~2.0 was generally accepted as "pure" for RNA and used in this study.

RT-PCR

Extracted viral RNA samples were first screened for the presence of M gene of Type A influenza virus by using RT-PCR. The samples detected positive for M gene were then tested further for subtyping using HA and NA gene specific primers in RT-PCR (Table 1). RT-PCR was performed with One *Taq* One-Step RT-PCR Kit (BioLabs Inc., New England).

For the identification of M, HA and NA protein genes of AIV, the initial denaturation was carried out at 98^{0}C temperature for 15 seconds. The elongation and final extension were carried out at 68^{0}C temperatures for 1 minute and 5 minutes respectively. Annealing temperature for M gene was set at 56^{0}C, for HA gene at 59^{0}C and for NA gene at 52.8^{0}C for 1 min (all cases). For electrophoresis of cDNAs, 1.5% agarose solution in 1X TAE buffer was used. The electrophoresis was carried out at 100V for 45 minutes; the gel was placed on the UV transilluminator, viewed and the picture was captured.

Table 1. Oligonucleotide primers used to detect M, HA and NA protein genes of AIV by using published primers

Target Gene	Primers Name	Sequence (5′-3′)	Amplicon size (bp)	Reference/ Gene Bank Accession No.
M gene	MF1	gaggtcgaaacgtacgttct	510bp	Ruba et al., 2015
(Type A)	MR1	ggccagcaccattctgttctc		
H5 gene	H5F	acaaagctctatcaaaacccaac	499bp	Chaharaein et al., 2009
	H5R	tacccataccaaccatctaccat		
H7 gene	H7F	caggcggaattgataaggag	409bp	Chaharaein et al., 2009
	H7R	tgccccattgaaactgaaag		
H9 gene	H9F	atcggctgttaatggaatgtgtt	221bp	Chaharaein et al., 2009
	H9R	tgggcgtcttgaatagggtaa		
NA gene	NAF	ttagcgggcaattcgtctct	1089bp	Ruba et al., 2015
	NAR	accacaaaaggatatgctgctc		

Results

Clinical History

Out of 32 birds extensively investigated in this study, typical clinical pathology observed were cyanotic comb and wattles in chicken; two were 33 weeks old layer birds (Figure 1) and third one was sub-clinically infected 4 weeks old broiler chicken. Signs of clinically infected layer birds showed depression and dizziness (Figure 1A), off feed, sudden death, cyanotic comb and wattle (Figure 1B), cyanotic shank (Figure 1C), respiratory distress, whitish to greenish faces with reduced egg production. The sub-clinically infected broiler bird was depressed, showed reduced food intake, isolated in shed and less active. The birds were sacrificed to evaluate extend of lesions in visceral organs. Mild coughing with discharged of thick mucus from mouth and nose were seen in sub-clinically infected broilers. Ducks and pigeons were died due to suspected cases of AI but did not reveal much change while examined at necropsy.

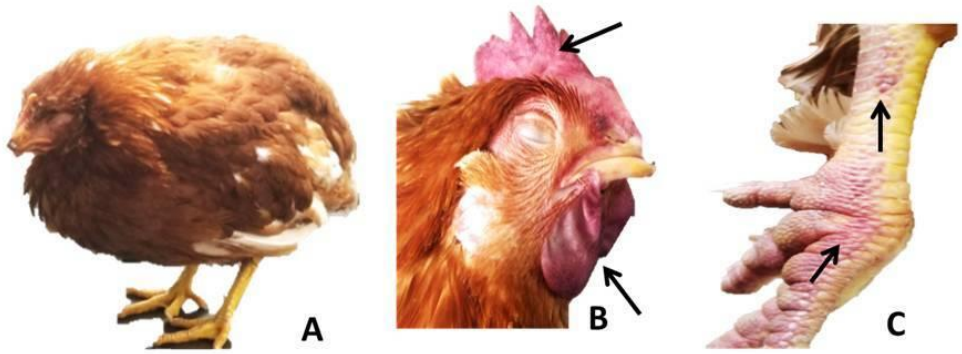

Figure 1: Clinical signs of infected layer chickens due to avian influenza virus showed marked depression, off feed and ruffled feathers (A). Typically, there was Cyanotic comb and wattles (B, arrows) with cyanotic shank (C, arrows).

Gross pathological lesions

The whole body of the clinically infected layer chicken appeared congested and hemorrhagic. Significant lesions observed at different organs were hemorrhagic tracheitis, hemorrhagic pancreatitis, congested and consolidated lungs, congested/ hemorrhagic coronary vessels and serosa of the intestine, streaky hemorrhage in the liver, congestions and hemorrhages in the spleen, hemorrhagic ovarian follicles, swollen and edematous kidney. Proventricular mucosa showed mild reddening and lacking of dominant hemorrhagic lesions. In sub-clinically infected broilers, only mild to moderate congestion and hemorrhages were seen in trachea (Figure 2D) with congested lungs (Figure 2A) but other organs appeared apparently healthy (Figure 2). Post mortem examination carried out onto the dead ducks showed wide spread hemorrhages and congestion in the visceral organs. Lungs were hemorrhagic and consolidated, there were hemorrhagic tracheitis, hemorrhages on pancreas and spleen and mild reddening of proventricular mucosa. Necropsy examination of pigeons also showed hemorrhagic tracheitis, congested and consolidated lungs, mild hemorrhages in intestinal serosa and coronary vasculature

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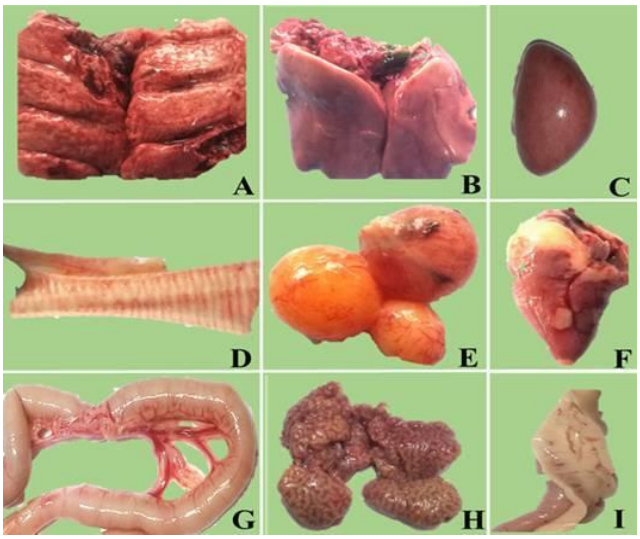

Figure 2: Visible lesions onto the visceral organs of sub-clinically infected layers were mild congestion and hemorrhages onto the tracheal mucosa (D). The lungs were weakly congested (A). Noticeable gross changes in the liver (B), spleen (C), ovarian follicles (E), heart (F), intestine (G), kidneys (H) and pancreas (I) were virtually absent or undetectable.

Histopathological findings

Widespread mild to moderate hemorrhages and congestion were seen in all of the visceral organs of clinically infected chickens. Hemorrhages and infiltration of reactive cells in the trachea, predominantly lymphocytes were seen (Figure 3A). Widespread congestion and hemorrhages with ruptured and distended alveoli (emphysema) were present in lungs. There was deposition of light pink color exudate in the distended lungs alveoli (Figure 3B). There was congestion and hemorrhages in the heart muscle (Figure 3C). In the liver, the central veins, sinusoids and capillaries were engorged with blood. Widespread hepatocellular degeneration was seen, which was characterized by intense acidophilia of the hepatocellular cytoplasm (Figure 3D). There was hemorrhages and congestions in the tips of the intestinal villi. The villi appeared fragile and sloughed off. Infiltration of reactive cells including lymphocytes and neutrophils was also prevalent in the intestinal mucosa along with hemorrhages. In the pancreas, hemorrhage was present in sub-capsular region with lymphocytic infiltration in the endocrine portion. There was necrosis and disappearance of lymphocytes in lymphoid follicles. Widespread congestions and hemorrhages were seen in the spleen. Glomerulonephritis, dilated Bowman's capsule, congestion and hemorrhages were seen in kidneys of layers clinically infected with H5 viral subtype.

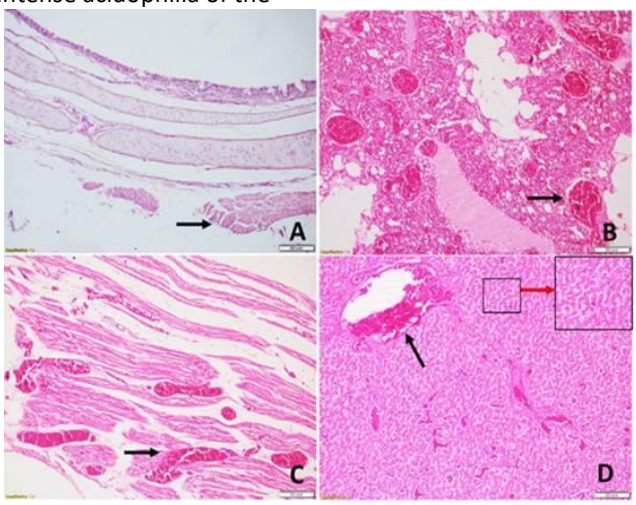

Figure 3: Histopathologic alterations in the visceral organs of chickens infected with the avian influenza A (H5N1) virus (10X). Hemorrhages (black arrow) and infiltration of reactive cells in the tracheal mucosa was seen (A). Emphysematous lungs with pink colored exudates within distended alveoli was seen along with massive congestion and hemorrhages (B, blue arrow). Widespread congestion (black arrow) and hemorrhages (blue arrow) was seen in the heart muscle (C). There was widespread degeneration of the hepatocytes in the cords (D, black

circle); The vasculature (veins, capillaries and sinusoids) of lungs (B, arrow), heart muscle (C, arrow) and liver (D, black arrow) were moderate to severely congested.

Ducks infected with AI virus showed wide spread congestions and hemorrhages throughout the body. In trachea, the congestions and hemorrhages were wider and profuse. There was infiltration of lymphocytes and neutrophils in tracheal mucosa. Lung's alveoli were sunk with copious pink color exudate and contain inflammatory cells including lymphocytes and neutrophils. Broilers sub-clinically infected with H9 subtype of AIV showed little changes like mild congestions and hemorrhages in the trachea, bronchiole, respiratory and intestinal mucosa. Mild congestion and hemorrhages were seen in the hepatic parenchyma. There was perihepatitis with infiltration of neutrophils. Severe congestion in renal parenchyma and glomerulitis were consistent findings in all broiler birds investigated.

Molecular detection and sub-typing of AIV

Extracted RNA from the trachea of the suspected birds was subjected to amplify by targeting fragments of M, HA and NA genes by RT-PCR. Firstly, the M gene specific amplification was carried out for the detection of type A AIV. Among the 32 birds tested, 18 birds were tested positive for M gene specific amplification and generated 510bp fragment in RT-PCR (Figure 4A)

For subtyping the AI viruses, amplification of the fragments of H5, H7, H9 and NA genes were carried out. Using RT-PCR, expected 499bp (Figure 4B), 221bp (Figure 5A) and 1089bp (Figure 5B) fragments of H5, H9 and NA genes were amplified respectively in RT-PCR settings. A total of 02 ducks and 04-layer chickens tested positive for H5 gene and 02 broilers for H9 gene. Using RT-PCR, H7 gene specific amplification of 409bp fragment was not generated in any birds. The NA gene specific amplification of 1089bp fragment was attained in 08 birds; those birds were also found to generate H5 and N9 gene specific response in RT-PCR. Sequencing and sequence analyzing of the fragment of amplified genes of AI viruses are in progress.

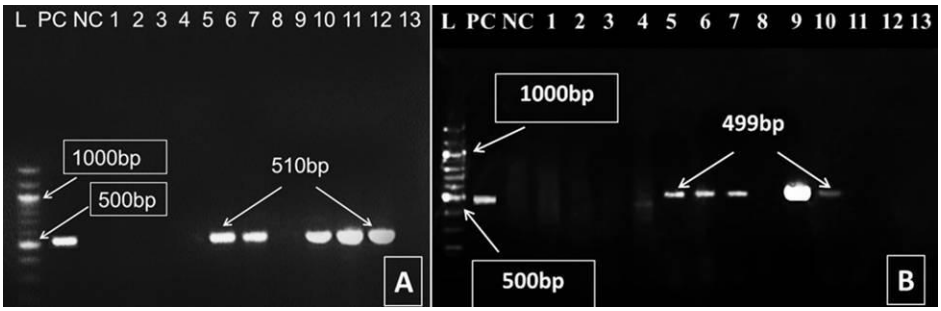

Figure 4: Partial amplification of M (left, A) and H5 (right, B) genes of AIV by using RT-PCR; where, lane L is for 100bp DNA ladder, lane PC is for positive control, NC is for negative control and lanes 1 to 13 are for test samples. In positive cases M gene specific 510 bp fragment (A, lane PC, 5, 6, 10, 11 and 12) and H5 gene specific 499bp fragment (B, lane PC, 5, 6, 7 and 9) were generated.

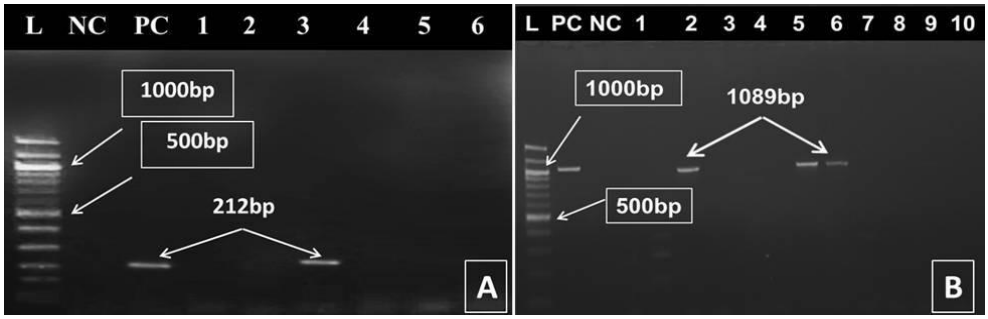

Figure 5: Partial amplification of H9 gene and NA gene of AIV by using RT-PCR; where, lane L for 100bp DNA ladder, PC is for positive control, NC is for negative control, lane 1 to 6 (A) and lane 1 to 10 (B) are for tests samples. In positive cases H9 gene specific 212 bp fragment (A, lane PC and 3) and NA gene specific 1089bp fragment (B, lane PC, 2, 5 and 6) were generated.

Discussion

Highly pathogenic avian influenza (HPAI) H5 viruses continue to impose a major biosafety and economic challenge to the global poultry sector due to their rapid transmissibility, tissue-tropic replication, and zoonotic potential (Lee et al., 2017). Bangladesh represents one of the regions where both HPAI H5N1 and LPAI H9N2 have co-circulated since their initial introduction (Biswas et al., 2008; Parvin et al., 2014), creating a multi-host ecosystem favorable for viral maintenance and mutations. The current study examined the spread

of AI viruses in Bangladeshi birds. Positive cases were identified, clinical investigation was conducted and virus identification was confirmed by RT-PCR.

The clinical and pathological features observed in the present study can be explained through established mechanisms of AIV pathogenesis. HPAI H5 viruses possess a polybasic cleavage site in the hemagglutinin (HA) protein, enabling ubiquitous activation by host proteases and facilitating systemic viral dissemination. This explains why earlier studies report widespread multiorgan hemorrhage and necrosis in H5-infected poultry (Lean et al., 2022). Although lesions observed here were comparatively milder, the distribution of tissue damage particularly in the trachea, lungs, liver, and spleen is consistent with the viral tropism of H5 viruses for endothelial and epithelial cells (Rahman et al., 2018; Parvin et al., 2019; Ameji et al., 2021).

The variation in lesion severity across poultry species being more pronounced in layer chickens, moderate in ducks, and comparatively mild in broilers and pigeons can be interpreted through host-specific innate immune responses and differences in viral replication. Layer chickens are known to exhibit higher susceptibility to HPAI because of a more intense pro-inflammatory cytokine response, which often leads to endothelial damage, vascular leakage, and hemorrhage. In contrast, domestic ducks typically mount a more regulated antiviral response and exhibit higher tolerance to viral replication, contributing to their role as silent reservoirs that shed virus for extended periods without manifesting severe pathology (Parvin et al., 2018; Lean et al., 2022; Makalo et al., 2022; Welchman et al., 2022). The comparatively mild changes in pigeons further align with previous findings that Columbiformes often exhibit lower susceptibility to AIV, and mortality in such species may be more closely associated with secondary infections rather than direct viral cytopathology.

The observed hemorrhages in respiratory and gastrointestinal organs correspond to viral replication within endothelial cells, leading to vasculitis, microvascular disruption, and leakage, as described in previous histopathological studies (Lean et al., 2022; Jannat et al., 2013; Parvin et al., 2019). Similarly, hepatic sinusoidal congestion and renal involvement (ME El-Makaki et al., 2010; El-Nagar et al., 2024) reflect the systemic nature of HPAI replication facilitated by the polybasic HA cleavage site. Pancreatic hemorrhage, which was prominent in layers and ducks, may be associated with the high density of sialic acid receptors (α2,3-linked), favoring viral attachment and replication in the exocrine pancreas.

RT-PCR amplification targeting M, HA, and NA genes remains the gold standard for AIV confirmation, as the M gene offers conserved target regions for influenza A detection (Parvin et al., 2019; Mumu et al., 2021). The subtype-specific primers used for HA and NA genes (Chaharaein et al., 2009; Ruba et al., 2015) have been widely validated in Bangladeshi poultry surveillance programs. The successful detection of both H5 and H9 subtypes in the present work reinforces earlier epidemiological findings that document their widespread endemicity in commercial farms and live bird markets (Ruba et al., 2015; Rahman et al., 2018; Nooruzzaman et al., 2019).

The continuing circulation of H5N1 in ducks, geese, chickens, and turkeys (Nooruzzaman et al., 2019; Ruba et al., 2015) highlights the interconnections of backyard flocks, commercial farms, and live bird markets, forming a transmission network that facilitates viral persistence. The identification of both H5 and H9 in the sampled population underscores the need for molecular surveillance that not only detects circulating subtypes but also characterizes genetic variation, reassortment patterns, and evolutionary trajectories (Parvin et al., 2020). Overall, the findings of this study emphasize that AIV infection dynamics in Bangladesh are shaped by a combination of viral pathogenic mechanisms, species-specific host responses, and ecological factors that support continuous viral maintenance. Sustained surveillance covering commercial poultry, ducks, pigeons, and backyard flocks is therefore critical for identifying emerging viral variants and mitigating the economic and public health risks posed by avian influenza.

Conclusion

This study investigated lesions in silently or sub clinically infected birds and detected pathology and subtype of AI viruses involved by using RT-PCR. Birds infected naturally with clinical form of HPAI (H5 subtype) showed wide spread hemorrhages in trachea, spleen, intestinal mucosa, pancreas and ovarian follicles as usual. Histopathological observations also revealed widespread congestion and hemorrhage in lungs, tracheal mucosa, heart muscle and liver. On the other way birds sub-clinically infected with LPAI viruses (H9 subtype) showed mild hemorrhages and congestion in tracheal mucosa and with mild congestion in lungs. Under microscopic investigation mild to moderate hemorrhages and congestions was seen in the visceral organs with variable level of mortality. For further analysis of NA subtype of AIV, sequencing and phylogenetic analysis of NA gene specific 1089bp fragment is in progress.

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