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Pathology, pathotype and phylogenetic analyses of a natural outbreak of highly pathogenic avian influenza (HPAI) virus in farming ducks of Bangladesh

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Abstract: *The present study was aimed at pathological investigation and genomic analysis of a highly pathogenic avian influenza (HPAI) virus from a natural outbreak of ducks in Bangladesh. Characteristics clinically signs observed were tremor of neck, head pressing on ground and diarrhoea with higher mortality. At necropsy hemorrhages and congestion were observed in upper respiratory tract, intestinal mucosa, ovarian follicles and meninges. Brush paint hemorrhages on pancreas were characteristics. Using histopathology, widespread hemorrhages were seen in parenchymatous organs. The lungs alveoli were drown with serofibrinous fluid. Focal necroses were common in pancreas and hepatocytes showed cytoplasmic vacuolation. Typing and sub-typing of the viruses were carried out by amplifying and sequencing matrix protein (M), hemagglutinin (HA) and neuraminidase (NA) genes. These genes were used in phylogenetic analyses and the studied viruses were belonging to clade 2.3.2.1. Presence of amino acid Glutamine (Q/238) and Glycine (G/240) (H5 numbering) and "PQGERRRKR*GLF" motif in the HA cleavage site indicating the affinity of the viruses to avian species and HPAI viruses respectively. Ducks in farming system are susceptible to clinical infection with HPAI. Continuous molecular and epidemiological surveillance onto the genomics of AIV in ducks is needed to monitor further mutation and emergence of pandemic AIV.*

Keywords: *Avian influenza, Duck, Farming system, HPAI, Natural outbreak.*

I. Introduction

Avian influenza (AI) is caused by the influenza virus of the taxonomic family Orthomyxoviridae that includes the genera influenza virus A, B and C. Among these genera only Type A influenza virus simultaneously infect several avian and mammalian species [1]. Wild aquatic birds, including ducks, are the natural reservoir of Type A influenza viruses and played significant role in the virus ecology and propagation. In wild ducks, the viruses replicate preferentially in the gastrointestinal tract, excreted at high concentration in feces and transmitted through oro-fecal route [2], [3]. Experimental inoculation of H5N1 AI viruses (AIV) replicate more efficiently in the upper respiratory tract than in the gastrointestinal tract of ducks [4].

From late 2003 and early 2005, H5N1 AI viruses causes severe fatalities not only in human but also in poultry and wild birds and remain circulated as endemic disease in Asia [4],[5],[6]. In Bangladesh, first case of H5N1 (HPAI) in poultry was reported in 2007 [7],[8] and since then the viruses have been detected in commercial and backyard chickens, domestic ducks, crow and migratory water fowls. Till date AI become endemic in Bangladesh and as a reservoir waterfowls played significant role in virus propagation [9],[10].

Therefore, it deems necessary to investigate natural case of HPAI in ducks, more specifically the pathology, pathotype, molecular biology and phylogenetic analyses of the viruses. This study was aimed at clinical and pathological investigation of a natural outbreak of HPAI in ducks of farming system at Mymensingh district, Bangladesh. Sub-typing of the viruses were carried out by sequencing and sequence analyses of the M, HA & NA genes. Phylogenetic and molecular evolutionary analyses with the amplified genes were carried out to know the pathotype of the virus towards avian or mammalian host types.

II. Materials And Methods

2.1 Clinical history and Sample collection

On 15 May 2013, an outbreak of AI in a duck farms (N=700) of Gouripur Upazila, Mymensingh was reported with a history of higher rate of morbidity (50%) and morbidity to mortality (85%). Following on farm investigation, the clinical signs observed were torticollis, tremor of neck, head pressing on ground, diarrhea, dizziness and respiratory distresses. Out of 300 dead ducks, five were examined, collected representative

samples to identify the pathology, pathotype and subtype of AI viruses by means of pathological investigation and genomic analyses of M, HA and NA genes of AIV.

2.2 Pathological investigation

Following necropsy pathological changes in lungs, liver, kidney, pancreas, intestine, heart, gizzard and skeletal muscles were recorded. The tissues from these organs were collected in 10% neutral buffered formalin for histopathological investigation. Portion of lungs and trachea were snap frozen and preserved at -80°C for molecular characterization. The formalin fixed tissues were processed, sectioned and stained with routine hematoxylin and eosin stain [11] and Goldner's trichrome staining [12]. The tissues were studied under low (10X) and high power (40X, 100X) microscopic field and images were captured using microphotographic system (Cell Bioscience, Alphaimager HP, California, USA).

2.3 Detection of M, HA & NA genes of AI virus

2.3.1 Extraction of Viral RNA

Portion of trachea and lungs were used for viral RNA extraction and detection of specific genes of AIV by using RT-PCR. Briefly 0.5 gm frozen tissues were crushed in liquid nitrogen and extracted viral RNA using commercial kit (Viral nucleic acid extraction kit II, Geneaid, USA). The quality and quantity of the extracted RNA was measured by using agarose gel electrophoresis and spectrophotometry (A260/A280).

2.3.2 RT-PCR detection of selected genes of AI virus

The partial M, HA and NA genes of AIV were amplified using SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA polymerase (Life Technologies, USA) kit. Primers for RT-PCR protocols were designed from sequences available in GenBank, synthesized from commercial source (1st base, Singapore) and fragments of M, HA and NA genes were amplified by RT-PCR (Table 1). The RT-PCR was carried out using One-Step RT-PCR System (SuperScript® III One-Step RT-PCR System, USA). The reaction was carried out in 50µl volume consisting of 2x master Mix, 1µl of each primer (20pmol/µl), 1µl of SuperScript III RT/Platinum Taq Mix, 1µl Rnase inhibitor, 5µl template RNA (50ng/reaction) and 17µl Nuclease free water. As negative control 5µl nuclease free H_2O was used instead of template RNA. The reaction was performed in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf, Germany).

RT-PCR for the amplification of selected genomes of AI virus (designed unified cycling parameter) was started with the reverse transcription at 45°C for 45mins. Then the initial denaturation was carried out at 95°C for 2mins followed by 40cycles of amplification reaction consisting of denaturation at 94°C for 30secs, annealing at 54°C for 30secs, elongation at 72°C for 3mins and final elongation at 72°C for 10mins. The RT-PCR was held at 4°C, the RT-PCR amplicons were analyzed by electrophoresis in 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) and images were captured.

2.3.3 Sequencing

Following gel cleaning of RT-PCR products, the cDNA was sequenced directly from the both direction using forward and reverse primers. Sequencing was done from commercial laboratory (1st Base, Singapore). The quality of raw sequence data were analyzed, edited and assembled with the programmes Chromas Lite, EditSeq and MegAlign for molecular and phylogenetic analyses.

2.4 Molecular evolutionary and phylogenetic analyses

The partial sequences of the HA and NA genes were subjected to Clustal W multiple sequence alignment using the Bioedit 7.1.5 program. Edited sequences were blasted using the NCBI database for sequence confirmation. Available sequences of other Bangladeshi isolates of AI were downloaded from GenBank for comparative study. Nucleotide sequence identity and divergence were carried out to check the homology of study viruses with other clade representative Bangladeshi isolates. Phylogenetic analyses were carried out using partial sequences of M, HA & NA genes by neighbor-joining (NJ) method with 1,000 replicates using software MEGA 6 version. For each phylogenetic analysis maximum composite likelihood substitution model was used. WHO/OIE/FAO recommendations on representative H5 strains from GenBank were included from particular clades. Geographic origin, year of isolation and sequence homology were also taken into consideration for construction of phylogenetic tree. The nucleotide sequences of M, HA and NA genes obtained in this study were made available in the GenBank database under the Accession Nos. **KT001068**, **KT359343** and **KT359344** respectively.

III. Results And Discussion

Clinical history

The natural outbreak of AIV in the infected farming ducks were characterized by torticollis, tremor of neck, head pressing on ground (Figure 1a), diarrhea, dizziness, respiratory distress with higher rate of morbidity (50%) and morbidity to mortality (85%). Out of 700 ducks in the farms, 350 were infected with a course of 15 days of outbreaks; of which 300 appeared dead. Five female Khaki Campbell ducks (7 months of age) were investigated extensively to find out the pathology, causes of death and pathotype of AIV involved with the disease process.

Pathological investigation

The skin and musculature of the affected birds appear congested and hemorrhagic. Petechial and echymotic hemorrhages were seen at the mucosa of upper respiratory tract. There were congestion in lungs (Figure 1b) and widespread hemorrhages in trachea, liver, spleen, heart, kidney and shanks. Swollen head and exudates in the orbital sinuses were noted. Ruptured ovarian follicles with hemorrhagic streaks on spleen and liver were seen. Characteristic brush paint hemorrhages were noticed on pancreas (Figure 1c). Head became swollen due to extensive hemorrhages, congestion and exudation in orbital sinuses [13]. Similar findings in a natural outbreak of AIV in layer chickens were also observed and the swollen head was due to hemorrhages, congestion and exudation in orbital sinuses [14].

Results of H&E staining showed widespread hemorrhages and congestions in the organs investigated. The lungs were hyperemic and congested with widespread hemorrhages in lungs alveoli, bronchioles and peribronchial tissues. The affected alveoli were emphysematous and ruptured with accumulation of serofibrinous exudates. Multifocal necroses were seen in pancreas, liver and kidney. Hemorrhagic pancreatitis, serositis in intestine and congested cardiac musculature with thickened epicardium were observed. Gizzard and skeletal muscles showed hemorrhages and congestion with infiltration of mononuclear cells. Widespread glomerulonephritis in kidney and individualization of hepatocytes in liver were seen.

Goldner's trichrome staining of infected tissue sections was carried out for distinctly visualized cellular responses in infected tissues. Lungs and trachea of affected tissues showed congestion, massive hemorrhages and lungs alveoli were drown with light blue color exudates (Figure 2a), indicating deposition of proteinaceous substances with fibrin. The exudates in lungs alveoli were dominated with lymphocytes. Exudation and cellular infiltration were also seen in the lumen of bronchiole. The deposition of exudates in lungs and bronchioles may be due to cytokine storm following infection with avian influenza virus [15]. Characteristics multifocal necroses were seen in pancreas, liver, kidney and the necrotic tissues showed intense staining reactivity to ponceau acid fuchsin (red color). More specifically cytoplasmic vacuolation was seen in infected hepatocytes (Figure 2b). Congestion and hemorrhages were also visible in kidney, pancreas, skeletal muscle, meninges and in intestine and was associated with vascular leakages. In general, there were massive hemorrhages in spleen and widespread hemorrhages in the serosa of intestine. Intranuclear inclusion bodies (Figure 2c) were distinctly visible in the nuclei of apparently healthy hepatocytes but was absent in hepatocytes containing pyknotic nuclei. This study provide evidence that lungs, heart and brain of ducks were severely inflamed and the death of infected individual could be due to cardiac, respiratory or nervous failure, alone or in combination. Previously the incidence of AI due to HPAI viral infection in layer chickens were described [14] and predominant lesions noted were severe pneumonia, congestion and hemorrhages in lungs. The massive inflammatory infiltration and exudation as seen in the lungs of infected chickens [14] and ducks indicated predominant pathology responsible for death of infected birds. Multifocal necroses were common in pancreas (Figure 2d), liver and kidney, these lesions could bear supportive evidence to identify HPAI viral infection in ducks and other avian species at necropsy and during histopathological investigation.

RT-PCR detection of M, HA and NA genes

The fragments of M (510bp), HA (1475bp) and NA (1089bp) genes of the viruses were detected with the RNA extracted from lungs of naturally infected ducks using RT-PCR (Figure 3). The oligonucleotide primers of the M, HA and NA genes as designed found to amplify specific band in gel documentation system which, however, was absent in control study. The cDNAs of these genes were gel cleaned and used in sequencing and molecular analysis.

Phylogenetic analyses and Nucleotide homology

Phylogenetic analyses of selected genomes (M, HA & NA genes) were undertaken to understand better the history and origin of AI viruses in ducks. Since the first outbreak of HPAI in February 2007, the viruses were well circulated in poultry with regular outbreaks. Results of sequencing and sequence analyses revealed that the Bangladeshi isolates of AI viruses were clustered into clade 2.2.2, 2.3.4 and 2.3.2.1. During 2007 to 2010, only clade 2.2.2 viruses were prevailing in Bangladesh. But later on 2011, two other clades (2.3.4 and 2.3.2.1) were introduced and detected in chickens, ducks, quails, feral crows and migratory birds [9], [16]. In South and South-East-Asian countries increased outbreaks of HPAI (H5N1) were detected and the outbreaks

were due to AI viruses belonging to clade 2.3.2.1. In 2012, all of the Bangladeshi isolates of HPAI were belonging to clade 2.3.2.1, indicating that clades 2.2.2 and 2.3.4 viruses were progressively merged to clade 2.3.2.1[9],[16],[17], [18]. In this study, the H5N1 virus (A/DK/BD/Gouripur 12/2013) investigated was also belonged to clade 2.3.2.1. Analyses of M, HA and NA genes of HPAI (A/DK/BD/Gouripur 12/2013) viruses (GenBank accession Nos. **KT001068**, **KT359343** and **KT359344** respectively) revealed that the investigating virus was clustered with the viruses circulated in domestic birds of Bangladesh, India, Bhutan and Pakistan (Figure 4, Figure 5, Figure 6) and are endemic in these regions.

Results of analyzing the nucleotide divergence of A/DK/BD/Gouripur 12/2013 viruses (Figure 7) showed maximum similarity (98.2%) with other isolates in clade 2.3.2.1 (A/ck/BD/12VIR-7140-11/12, A/ck/BD/12VIR-7140-17/12 and A/dk/BD/ 17753/12) and least similarity (90.4-91.1%) with clade 2.2.2 viruses. In migratory water fowls, 99% nucleotide homology were reported in AIV belonging to clade 2.3.2.1 and also noted clear distance in isolates from clade 2.2.2 viruses [16].

Molecular genetic analyses

Molecular evolutionary analyses of deduced amino acid (aa) sequence of HA protein was carried out targetting sequences at the cleavage site, receptor binding pockets, glycosylation site and antigenic site. H5 numbering was followed for cleavage site, glycosylation sites, antigenic sites and hemadsorption sites [19], while H3 numbering for receptor binding sites [20]. Analyses of the N-X-T/S motif (X can be any aa except proline) revealed that all Bangladeshi HPAI isolates have six glycosylation sites (^{27}NST^{29},^{39}NVT^{41}, ^{181}NNT^{183} ,^{302}NSS^{304}, ^{500}NGT^{502},^{559} NGS^{561}) except CK/BD/11rs 1984-37/2011 that bear five glycosylation sites. Similar findings were also reported earlier [18] with minor variations (^{302}NSS^{304} /^{286}NNS^{288}). The reason of this variation could be due to mutations at these sites. As partial sequence, the amplified region of studied H5 gene containing four glycosylation sites (^{156}NSS^{158},^{170}NNT^{172}, ^{181}NNT^{183} ,^{302}NSS^{304}, Table 2). Two other glycosylation sites were placed in two terminals of the genes, not amplified in this study and therefore, was not, included in comparison. Globally variable glycosylation sites were also noted in different isolates of H5N1, H9N2 and H7N3 viruses (Table 5). Number and location of glycosylation sites are necessary for virus host interaction and its alteration influences the adaptation profile of AI viruses to poultry and could have altered their pathogenicity and antigenicity. This alteration also helps viruses to the evasion from host antibody response [21].

A detailed aa comparison of the local isolate (A/DK/BD/Gouripur 12/2013) with other representative sequences was performed to analyze specific conserved regions or motifs related to viral pathogenicity and virulence. The aa sequences of HA protein exhibited a ^{337}PQRERRRK-R*GLF^{349} motif which is in contrast with ^{337}KRRKR*GLF^{344} and ^{333}PARSSR*GLF^{341} motif of H7N3 and H9N2 isolates represents the cleavage site (Table 5). The aa sequence of HA cleavage site stated that all clade 2.2.2 viruses have ^{337}PQGERRRKKR*GLF^{349} motif whereas clade 2.3.4 and clade 2.3.2.1 have ^{337}ALREKRRK-R*GLF^{349} and ^{337}PQRERRRK-R*GLF^{349} motif (Table 2). One deletion at the cleavage site (345) was observed in clade 2.3.4 and clade 2.3.2.1 viruses. All studied HPAI Bangladeshi isolates have multiple basic aa at the cleavage site which is the characteristics of HPAI viruses [18], whereas, LPAI viruses have mono or di-basic aa (Table 5).

Deduced aa sequences of all HPAI isolates were screened for the presence of residues [22] that are considered as signature of pandemic human influenza viruses (Table 2). All HPAI isolates including A/DK/BD/Gouripur 12/2013 had Q and G at positions 238 and 240 (H5 numbering) indicating that the viruses are primarily selective to avian type (α-2, 3 linked sialic acid receptor) [16], [19]. Literature available indicated that the circulating LPAI virus (H9N2) are low pathogenic to avian species but tends to have affinity to bind with mammalian cell receptor as they have mutation in aa position 234 (Q/L) of RBP. The reassortment of HPAI (H5N1) and LPAI (H9N2) viruses in poultry of Bangladesh were reported earlier [18], where H5N1 virus acquired the PB1 gene from H9N2 viruses.

Based on immunological characterization and sequencing of HA molecule of escape mutants [19], [23], the three dimensional structure of HA molecule has three antigenic sites (H5 numbering) these are: site 1 from residues 136 to 141, site 2 from residues 152 to 153 and site 3 from residues 125 to 129. In this study, the antigenic site 1 of the virus (A/DK/BD/Gouripur 12/2013) has two substitutions (L138Q and T140N) and at site 3 there is one substitution (S129L) but the antigenic site 2 did not reveal any mutation (Table 3). The HA clade of 2.3.2.1 has similar substitutions as reported by [18]. Alterations of antigenic sites are associated with phenotypic changes which is associated with changes of virus affinity towards cell receptor or virulence [24].

Most of the pandemic influenza viruses (Spanish flu, Asian flu, Hong Kong flu, swine flu) reassort genes from avian viruses to infect mammals [25], [26] and reassorment of either HA and NA genes of AI viruses could generate pandemicity of AI viruses. The neuraminidases (NA) gene of avian viruses have separate sialic acid binding site which is called hemadsorption site (HB). The HB site has got a shallow pocket located near the deep catalytic site and formed by three surface peptide loops: 1st loop 345 to 350; 2nd loop 377 to 380; 3rd loop 412 to 414 (N1 numbering). These HB sites enhance catalytic efficiency of NA and are also associated

with NA inhibitor drug resistance. Therefore, changes of receptor binding specificity of hemagglutinin and alteration of HB sites is also necessary for the emergence of pandemic influenza viruses [27]. In this study, analysis of hemadsorption sites of NA gene of AI viruses (Table 4) with those of Bangladeshi isolates showed no substitution at 1st and 3rd loop where 2nd loop had a R379W substitution [10]. The results of sequencing of HA and NA genes showed that there were several mutations in the HPAI viruses (Table 2, 3, 4). Important mutation was noticed in NA gene at L138Q, T140N and S129L sites but the viruses are still avian type.

IV. Figures And Tables

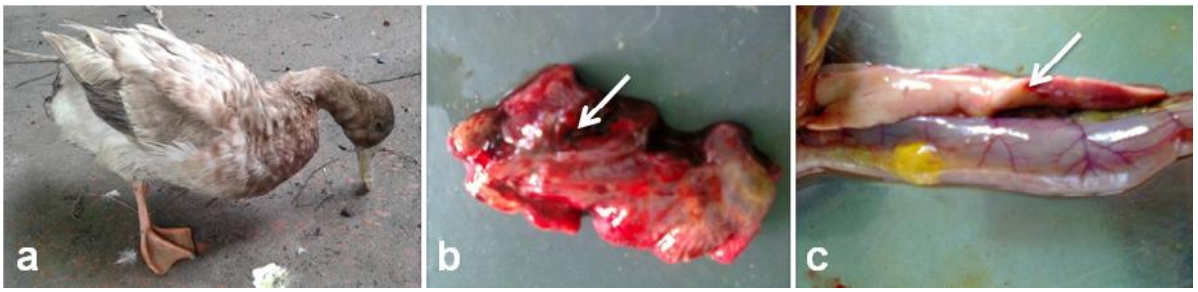

Fig. 1 The infected ducks showed atypical signs like head placing on ground (a). At necropsy the lungs appeared severely congested and consolidated (b). Brush paint haemorrhages were common in the pancreas of infected ducks (c).

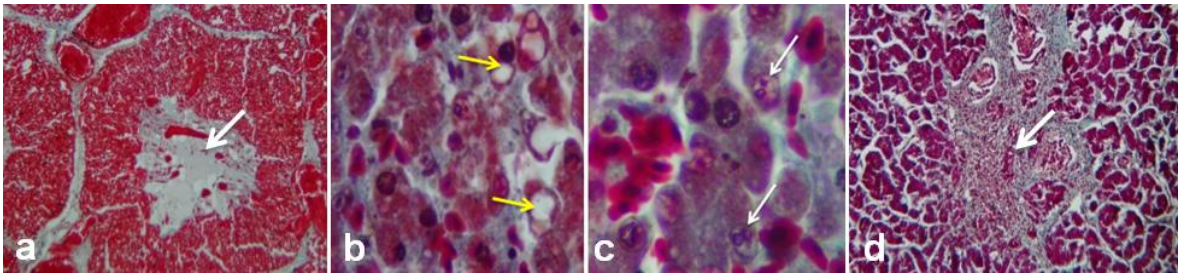

Fig. 2 Section of lungs (a), liver (b, c) and pancreas (d) stained with Goldner's trichrome. The section of lungs (10X) showed widespread hemorrhage and congestion, massive serofibrinious exudation (a, arrow) in distended and ruptured alveoli. There were widespread hemorrhages and congestion in liver. Cytoplasmic vacuolation (b, yellow arrow) and intranuclear inclusion bodies (c, white arrow) in hepatocytes were seen (100X). Multifocal necrosis was seen in pancreas and the necrotic tissues were infiltrated with lymphocytes (d, 40X).

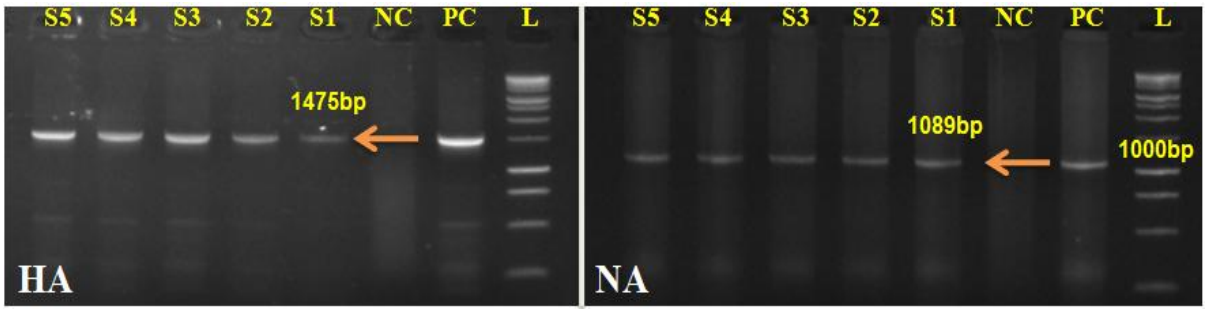

Fig. 3 Amplifications of HA (1475bp) and NA (1089bp) genes of AI virus from lung tissues by using RT-PCR. Lane L=1KB DNA size marker, PC= Positive control, NC= Negative control, S1-S5 = Test samples.

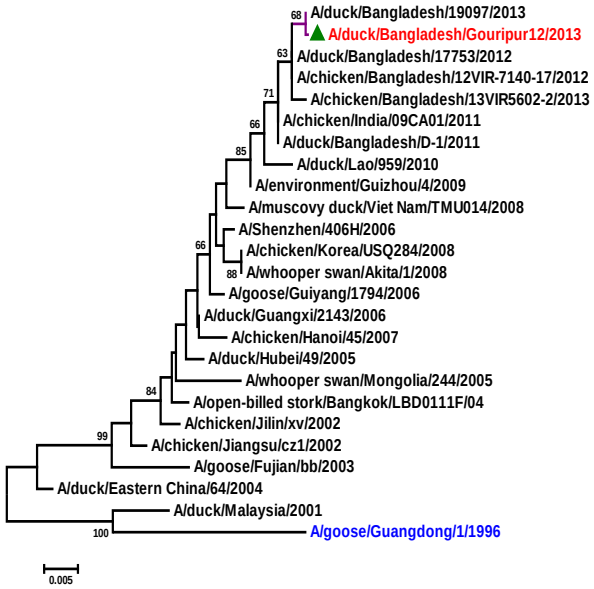

Fig. 4 Neighbor-joining evolutionary tree based on partial M gene (391bp) sequence of H5N1 HPAI virus along with different representative AI viruses. Numbers at the branch nodes indicate Bootstrap values (1000 replications) above 60%. Root virus A/goose/Guangdong /1/1996 was indicated by blue taxon color. Red color taxon name with green triangle denoted the study virus.

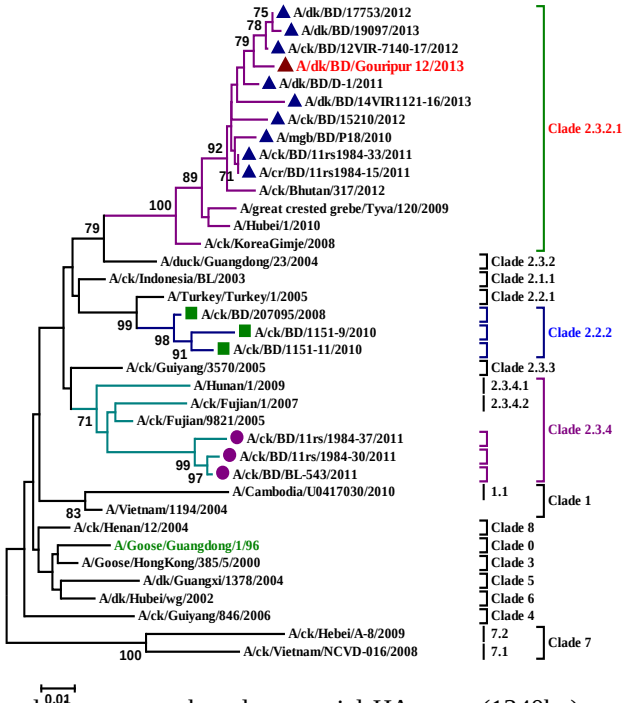

Fig. 5 Neighbor-joining evolutionary tree based on partial HA gene (1340bp) sequence of H5N1 HPAI virus along with different clade representative viruses. Bootstrap values (1000 replication) above 70% are shown next to the nodes. Bangladeshi isolates are indicated with symbols: closed rectangle – clade 2.2.2; closed circle –

clade 2.3.4; closed triangle – clade 2.3.2.1. Abbreviations: ck, Chicken; dk, Duck; cr, Crow; mgb, migratory bird; BD, Bangladesh.

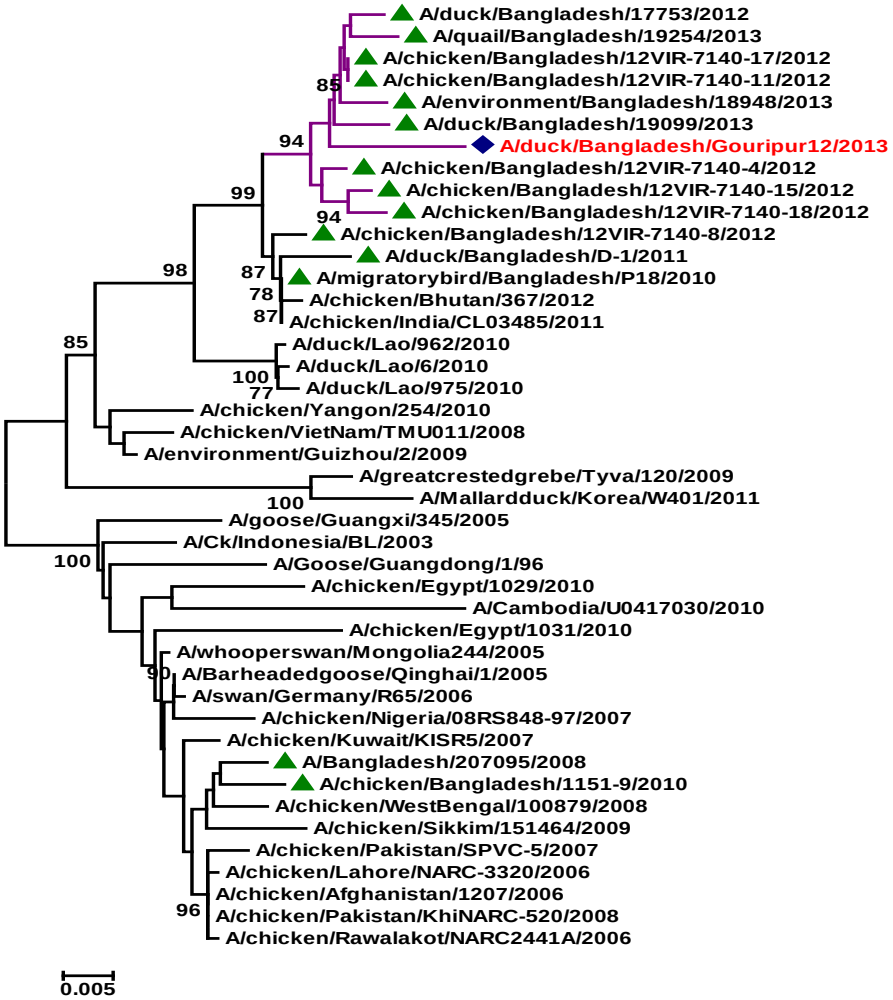

Fig. 6 Neighbor-joining evolutionary tree based on partial NA gene (993bp) sequence of H5N1 HPAI virus along with different representative avian influenza viruses. Numbers at the branch nodes indicate Bootstrap values (1000 replications) above 70%. Selected isolate from ducks is marked with blue closed tetrangle and the taxon name showed red in color, other Bangladeshi isolate shown in closed green color.

Percent Identity

	1	2	3	4	5	6	7	8	9	
1		99.5	99.5	93.1	92.6	92.2	92.2	92.2	91.1	1
2	0.5		99.6	92.8	92.3	91.7	91.7	91.7	90.4	2
3	0.5	0.4		92.8	92.3	92.0	92.0	92.1	90.7	3
4	6.6	7.0	7.0		98.0	92.6	92.6	92.6	92.0	4
5	7.2	7.6	7.6	2.0		92.5	92.5	92.5	92.0	5
6	7.7	8.3	7.9	7.6	8.0		100.0	100.0	98.2	6
7	7.7	8.3	7.9	7.6	8.0	0.0		100.0	98.2	7
8	7.6	8.2	7.8	7.6	8.0	0.0	0.0		98.2	8
9	8.7	9.5	9.2	8.5	8.5	1.8	1.8	1.8		9
	1	2	3	4	5	6	7	8	9	

Divergence

1 A Bangladesh 207095 08
2 A chicken Bangladesh CD-08(09)BL-418 09
3 A chicken Bangladesh 1151-11 10
4 A chicken Bangladesh 11rs1984-30 11
5 A chicken Bangladesh 11rs1984-37 11
6 A chicken Bangladesh 12VIR-7140-11 12
7 A chicken Bangladesh 12VIR-7140-17 12
8 A duck Bangladesh 17753 12
9 A duck Bangladesh Gouripur 12 13

Fig. 7 Percent identity and divergence of HA gene of A/Duck/ Bangladesh/ Gouripur 12/13 and other available clade representative Bangladeshi isolates of H5N1 HPAI viruses. Study viruses showed 90.4-98.2% homology with other isolates.

Table 1. Oligonucleotide primers designed to detect M (510bp), HA (1475bp) and NA (1089bp) genes of AI virus in farms duck from a natural outbreak

Target Gene	Primer Name	Sequences (5'-3')	Primer position (bp)	Amplicon size (bp)	GeneBank Accession No.of source sequence
M gene (Type A AIV)	MF1	GAGGTCGAAACGTACGTTCT	41-61	510	GU727664.1
	MR1	GGCCAGCACCATTCTGTTCTC	571-551		
	MF2	GAGAACAGAATGGTGCTGGCC	551-571	430	
	MR2	CTGTGGATGT TGACGATGGTC	971-961		
HA gene	HAF	ACACAACGGGAAACTCTGC	170-188	1475	AB741558
	HAR	GTACTAGGGAACTCGCCACTGT	1623-1644		
NA gene	NAF	TTAGCGGGCAATTCGTCTCT	193-212	1089	KF597849
	NAR	ACCACAAAAGGATATGCTGCTC	1260-1281		

Table 2. Molecular analyses of Bangladeshi H5N1 HPAI isolates of AI viruses. All of the selected isolates of respective clades have similar HPAI motif. Selected isolates including A/DK/BD/Gouripur 12/2013 had Q and G at positions 238 and 240 (222 and 224 in H5 numbering) indicating that the viruses are primarily specific for α-2,3 linked sialic acid receptor (avian type)

Selected strain	HA clade	Cleavage site (337-349)	Left edge of RBP (236-241)	Right edge of RBP (146-150)	Total Glycosylation site (N-X-T/S motif)
BD/207095/ 08	2.2.2	PQGERRRKKR*GLF	NG**Q**S**G**R	GVSSA	6
Ck/BD/1151-11/10	2.2.2	PQGERRRKKR*GLF	NG**Q**S**G**R	GVSSA	6
Ck/BD/CD-08(09)BL-418/ 09	2.2.2	PQGERRRKKR*GLF	NG**Q**S**G**R	GVSSA	5
Ck/BD/11rs1984-30/ 11	2.3.4	ALREKRRK-R*GLF	NG**Q**S**G**R	GVSSA	6
Ck/BD/11rs1984-37/ 11	2.3.4	ALREKRRK-R*GLF	NG**Q**S**G**R	GVSSA	6
Ck/BD/12VIR-7140-11/12	2.3.2.1	PQRERRRK-R*GLF	NG**Q**S**G**R	GVSSA	6
Ck/BD/12VIR-7140-17/12	2.3.2.1	PQRERRRK-R*GLF	NG**Q**S**G**R	GVSSA	6
DK/BD/17753 /12	2.3.2.1	PQRERRRK-R*GLF	NG**Q**S**G**R	GVSSA	6
***DK/BD/Gouripur 12/13**	2.3.2.1	PQRERRRK-R*GLF	NG**Q**S**G**R	GVSSA	4 (due to partial sequence)

Abbreviations: RBP, receptor binding pocket; CK, chicken; DK, duck; BD, Bangladesh.

Table 3. Analyzing the antigenic sites on HA of HPAI viruses and was carried out by using Cluster W method in Bioedit 7.1. Special emphasis was given to identify aa substitutions on HA protein including Q138L, R140T, T140N, S141P, P141S, E126S, A127T and S129L sites. Out of three sites investigated there were consistent point mutation in site 1 of clade 2.2.2, 2.3.4, 2.3.2.1 viruses. Highest rate of mutation were seen at position 138 and 140 of HA protein strand

Selected strain	HA clade	Site 1 (136-141)	Site 2 (152-153)	Site 3 (125-129)
BD/207095/ 08	2.2.2	PYQGRS	KK	HEASS
Ck/BD/CD-08(09)BL-418/ 09	2.2.2	PYQGRS	KK	HEASS
Ck/BD/ 1151-11/ 10	2.2.2	PYQGRS	KK	HEASS
Ck/BD/ 11rs1984-30/ 11	2.3.4	PY**L**G**TP**	KK	H**S**AS**L**
Ck/BD/ 11rs1984-37/ 11	2.3.4	PY**L**G**TP**	KK	HE**TSL**
Ck/BD/ 12VIR-7140-11/12	2.3.2.1	PYQG**N**S	KK	HEAS**L**
Ck/BD/ 12VIR-7140-17/12	2.3.2.1	PYQG**N**S	KK	HEAS**L**
DK/BD/17753 /12	2.3.2.1	PYQG**N**S	KK	HEAS**L**
***DK/BD/Gouripur 12/13**	2.3.2.1	PYQG**N**S	KK	HEAS**L**

Abbreviations: CK, chicken; DK, duck; BD, Bangladesh.

Table 4. Hemadsorption site (HB) of NA genes of Bangladeshi isolates of HPAI virus by using Cluster W method in Bioedit 7.1. Substitution of amino acids in 2nd loop (R379W) of NA protein indicated that the viruses belonged to avian type. Substitution of amino acids was absent in 1st and 3rd loop of NA protein

Hemadsorption sites (HB) of NA protein of AI viruses	**Specification of amino acids in various loops**		
	1st loop (345-350)	**2nd loop (376-380)**	**3rd loop (411-414)**
BD/207095/08	STNSRS	ITD**R**	PKE
CK/BD/1151-9/10	STNSRS	ITD**W**	PKE
CK/BD/1151-11/10	STNSRS	ITD**W**	PKE
Ck/BD/ 12VIR-7140-11/12	STNSRS	ITD**W**	PKE
Ck/BD/ 12VIR-7140-17/12	STNSRS	ITD**W**	PKE
CK/BD/12VIR-7140-7/12	STNSRS	ITD**W**	PKE
CK/BD/12VIR-7140-16/12	STNSRS	ITD**W**	PKE
DK/BD/17753 /12	STNSRS	ITD**W**	PKE
***DK/BD/Gouripur 12/13**	STNSRS	ITD**W**	PKE

Abbreviations: CK, Chicken; DK, Duck; BD, Bangladesh**.**

Table 5. Molecular analyses of the aa sequences of HA protein of HPAI (H5N1) isolate and compared the corresponding aa sequences of LPAI (H7N3 and H9N2) viruses. The analysis was carried out by using Cluster W method in Bioedit 7.1 and bold letter indicated mutation sites in the protein. The cleavage site (337-349) of the studied virus (DK/BD/Gouripur 12/13) and HPAI viruses circulating globally containing polybasic aa, which, however were mono or di-basic in case of LPAI viruses. The left edge of HPAI virus has got aa Q (238) and G (240) showed preferred binding affinity to α 2, 3 sialic acid receptor

Selected isolates	**Cleavage site (337-349)**	**Left edge of RBP (236-241)**	**Right edge of RBP (146-150)**	**Glycosylation sites (N-X-T/S motif)**
***DK/BD/Gouripur 12/13**	PQRERRRK-R*GLF	NG**Q**S**G**R	GVSAA	*4 (partial sequence)
CK/BD/12VIR-7140-7/ 12	PQRERRRK-R*GLF	NG**Q**S**G**R	GVSAA	6
CK/Fujian/ 9821/05	PLRERRRK-R*GLF	NG**Q**S**G**R	GVSSA	5
CK/Egypt /1029/ 10	PQGERRRKKR*GLF	NG**Q**S**G**R	GVSSA	5
CK/Israel/ 65/ 10	PQGEGRRKKR*GLF	NG**Q**S**G**R	GVSSA	4
CK/Korea/ Gimje/ 08	PQRERRRK-R*GLF	NG**Q**S**G**R	GVSSA	6
CK/Thailand/CU-354/ 08	PQRERRRKKR*GLF	NG**Q**S**G**R	GVSSA	5
Goose/ Guangdong/1/96	PQRERRRKKR*GLF	NG**Q**S**G**R	GVSSA	5
great crested grebe/Tyva120/09	PQRERRRK-R*GLF	NG**Q**S**G**R	WVSAA	6
Vietnam /1194 /04	PQRERRRKKR*GLF	NG**Q**S**G**R	GVSSA	5
ws/Mongolia/ 244/ 05	PQGERRRKKR*GLF	NG**Q**S**G**R	GVSSA	5
Ck/Indonesia/BL/ 03	PQRERRRKKR*GLF	NG**Q**S**G**R	GVSSA	5
Kar/NARC/ 100/04 (H7N3)	^{337}KRRKR*GLF^{344}	^{233}NG**Q**S**G**R^{238}	^{142}GATSS^{146}	2
DK/Guangdong/1/96 (H7N3)	^{333}K--TR*GLF^{341}	^{233}NG**Q**S**G**R^{238}	^{142}GTTSA^{146}	3
CK/BD/VP01/06 (H9N2)	^{333}PAkSSR*GLF^{341}	^{232}NG**L**I**G**R^{237}	GTSKS	5
Av/ SA/910135/06(H9N2)	^{333}PARSSR*GLF^{341}	^{232}NG**L**I**G**R^{237}	GTSKS	5
CK/Du/ 463/ 03(H9N2)	^{333}PARSSR*GLF^{341}	^{232}NG**L**I**G**R^{237}	GTSKA	3
CK/Em/R66/02(H9N2)	^{333}PARSSR*GLF^{341}	^{232}NG**Q**L**G**R^{237}	GTSKA	5
CK/Pa/UDL-01/08(H9N2)	^{333}PAKSSR*GLF^{341}	^{232}NG**L**I**G**R^{237}	GTSKS	5

Note: RBP, receptor-binding pocket; DK, duck; CK, chicken, Av, avian; ws, whooper swan, BD, Bangladesh; SA, Saudi Arabia; Em, Emirates ;DU, Dubai; Pa, Pakistan; Kr, Karachi. Numbering according to H5 for H5N1, H9 for H9N2, H7 for H7N3 –retrieved from GenBank

V. Conclusion

Results of clinico-pathological investigation indicated high morbidity and mortality of infected ducks, mild hemorrhages in upper respiratory tract and intestine, brush paint hemorrhages in pancreas, hemorrhagic and serofibrinous exudation in lungs and intranuclear inclusion bodies with cytoplasmic vacuolation in hepatocytes; these changes were suggestive for infection with HPAI. The studied virus had aa Q and G at positions 238 and 240 respectively (222 and 224 in H5 numbering) indicating that the viruses are primarily specific α-2,3 linked sialic acid receptor. The RT-PCR protocols designed in this study selectively detect H5N1 viral infection from a natural outbreak of HPAI and can be used in future. Constant monitoring onto the generation of new AI viruses having affinity to both mammalian and avian type receptor is needed to trace pandemic AI viruses at early onset.

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