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BROILERS OF LIVE BIRD MARKETS AT MYMENSINGH CITY CARRYING H9N2 AVIAN INFLUENZA VIRUS IN THEIR RESPIRATORY TRACT

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ABSTRACT

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Avian influenza (AI) caused by Type A influenza virus is a global zoonosis, infecting vast majority of mammalian and avian species. Broilers are meat type birds and randomly reared and sold by the farmers in Bangladesh with poor biosecurity. This study was aimed to identify the Type and subtypes of AI viruses in the broilers of two live bird markets, Mymensingh. A total of 10 birds from each of the market were randomly selected, investigated by clinical, pathological, reverse transcriptase polymerase chain reactions (RT-PCR), sequencing and sequence analysis. Out of 20 birds investigated, 06 were sick, 02 were dead and 12 were apparently healthy. Clinically, the sick/dead birds did not reveal any changes typical to AI. During necropsy, the sick/dead birds showed congested lungs and moderate hemorrhages in the trachea. Such lesions was absent in the lungs of apparently healthy birds. Following histopathological examination interstitial pneumonia with bronchitis was seen in sick/dead birds. The RT-PCR protocol was adapted to identify matrix protein gene of Type A influenza virus and amplified 430bp fragment is even cases. To identify the sub types of AI viruses involved, hemagglutinin (HA) and neuraminidase (NA) gene specific RT-PCR was carried out. 1475bp and 1089bp amplicons specific to HA and NA genes of AI viruses were generated in 07 cases. The cDNAs of HA and NA genes were sequenced, edited and revealed that the AI virus circulated in the live bird market of Mymensingh city is H9N2 subtype. Two sick, one dead and four apparently healthy birds found to carry H9N2 AI virus. The H9N2 virus is naturally low pathogenic for poultry, has got public health significance, and may donate partial or even whole cassette of internal genes to generate novel human-lethal reassortants of AI viruses; this was main concern for AI viral outbreak investigation in this study. It needs to examine large number of samples from wider sources to trace the rate of mutation and subsequent reemergence of pandemic AI viruses.

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INTRODUCTION

Poultry industry becomes an emerging agribusiness since 1980 in Bangladesh (Islam and Ali, 2009). Presently, there are 53112, 18222 and 6546 commercial broilers, layers and duck farms respectively in Bangladesh. From this sector, an estimated 1.6millions Kg of meat and 23.5millions of eggs contributed in the meal of Bangladeshi people (Hamid *et al.*, 2017). At present, the poultry population in Bangladesh is estimated to be around 304.17 million where chicken population is about 255.31 million and duck population is 48.86 million (DLS, 2015). For economic solvency, the unemployed and low income group people are trying to improve their financial strength through broiler farming. Rearing of birds in farming condition is popularly known as Farm birds; mainly considered as chickens (broiler, layer and native), duck, goose, pigeon and quail. Scientific breeding, feeding, management, disease diagnosis and control are the key point of success in broiler farming but the main hindrance is their routine morbidity and mortality (around 30%) due to a number of infectious diseases (Das *et al.*, 2005). Newcastle disease (ND), Infectious bursal disease (IBD), Avian influenza (AI), Marek's disease (MD), Fowl pox (FP), Pullorum disease (PD), Fowl cholera (FC), Collibacillosis (CB), Fowl typhoid (FT) etc. are the dominant infectious diseases responsible for higher rate of morbidity and mortality of poultry in Bangladesh. Now a day AI is a commonly occurring malady in poultry industry in Bangladesh including broilers.

Influenza is an intense ongoing potential threat to human and animal health. Each year, millions of people are infected globally with seasonal influenza. Additionally, they may also be exposed to avian (H5, H6, H7, H9, and H10) and swine (H1 and H3) influenza subtypes (García-Sastre *et al.*, 2014). During previous human influenza pandemics, the causative virus subtypes emerged from avian influenza viruses that had crossed the species barrier (Kawaoka *et al.*, 1989; Webster *et al.*, 1992; Taubenberger *et al.*, 1997; Yoon *et al.*, 2014). To date, avian influenza subtypes H5N1, H7N9, and H9N2 have become endemic in poultry in Eurasia and are the leading candidates that have the potential to transmit to humans and cause lethal infection (Fauci 2006; Watanabe *et al.*, 2014). Each of these influenza subtypes has domestic poultry as an intermediate host and is predominantly isolated from live-bird markets, which are a proven risk factor for zoonotic transmission between birds and humans.

Initially, AI was reported in Italy during 1878, the disease caused uncountable mortality of poultry, which was then termed as "Fowl plague" (Lupiani *et al.*, 2009). The disease was recognized as filterable virus in 1901 and the causative agent was formally designated as influenza A virus (Lupiani *et al.*, 2009; Alexander, 2000). Rather than the highly pathogenic form, the low pathogenic AI viruses have been successively detected in various countries since the mid-19th century. The first isolate from chickens was established in Germany in 1949 [A/chicken/Germany/1949(H10N7)] without being recognized and defined the specific subtype till 1960. As for the H9N2 subtype, the virus spread becomes more and more extensive at around 1990s, resulting in continuous viral circulation in many countries in Asia, Middle East and North Africa (Alexander, 2007). The H9N2 AI virus could cause damage to birds with direct infection, co-infection and immunosuppression (Sun *et al.*, 2015; Zhang *et al.*, 2008). Apart from that, H9N2 viruses not only infect mankind, but also provide partial or even fully reassortment of internal genes to emerge human-pandemic H5N1, H7N9, H10N8 and H5N6 reassortants (Gu *et al.*, 2017; Guan *et al.*, 2000; Shen *et al.*, 2016; Zhang *et al.*, 2016; RahimiRad *et al.*, 2016), posing a substantial threat to public health. Therefore, the present research work was conducted for the clinicopathological investigation of AI in broilers at live bird markets and detection of the subtypes of AI virus existed in the upper respiratory system by using RT-PCR and sequencing.

MATERIALS AND METHODS

Clinicopathological investigation

Broilers of two live bird markets of Mymensingh city were investigated (n=20) and necropsised in the Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University (BAU), Mymensingh during March to May, 2016. A total of 06 sick, 02 dead and 12 apparently healthy broilers were collected from KR market, Bangladesh Agricultural University (BAU) and Kewatkhali live bird market, Mymensingh city. 10 birds (four sick/dead and six apparently healthy) from each of the market were investigated. During live bird market investigations, the sick birds (N=06) showed mild gasping, reduced feed

and water intake, slightly loose feces and dizziness. The sick/ dead (N=08) and apparently healthy (N=12) broilers were brought to the Department of Pathology, necropsied and gross changes observed were recorded. Following necropsy, portion of tracheas was snap frozen. Portion of lungs, liver, heart, pancreas, skeletal muscle, duodenum, trachea and spleen were collected and fixed in 10% neutral buffered formalin for histopathological studies. Formalin fixed tissue samples were processed and stained with H&E stain (Luna, 1968). Briefly the tissues were embedded in paraffin, 4-5µm thick sections were float on lukewarm water (42°C) containing gelatin and the floating tissues were pick up onto clean slides. The tissues on to slides were stained with hematoxylin and eosin (H&E) and were mounted using DPX. The sections on to the slides were examined at low and high power microscopic field. The images were captured and labelled for better understanding the changes.

RT-PCR detection of Type A influenza viruses

Portion of trachea (N=20) of the broilers were used in viral RNA extraction and detection of matrix protein genes of AI virus by using RT-PCR. Briefly 50mg of frozen tracheal tissues were crushed in liquid nitrogen and extracted viral RNA using QIA amp *Viral RNA Mini Kit* (QIAGEN, Germany). The quality and quantity of the extracted RNA was measured by using agarose gel electrophoresis and spectrophotometry (A_{260}/A_{280}). The matrix (M) protein gene of AI viruses was targeted to amplify in an RT-PCR setting (Table 1).

Table 1. Oligonucleotide primers used to amplify M (430bp), HA (1475bp) and NA (1089bp) genes of AI virus by using RT-PCR (Ruba et al., 2015)

Target Gene	Primers Name	Sequences (5'-3')	Primer position (bp)	Amplicon size (bp)
M gene (Type A AIV)	MF2	gagaacagaaatggtgctggcc	551-571	430
	MR2	ctgtggatggtgacgatggtc	971-961	
HA gene	HAF	acacaacgggaaactctgc	170-188	1475
	HAR	gtactagggaaactcgccactgt	1623-1644	
NA gene	NAF	ttagcgggcaattcgtctct	193-212	1089
	NAR	accacaaaaggatgatgctgctc	1260-1281	

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) and 17µl Nuclease free water. As negative control 5µl nuclease free H₂O was used instead of template RNA. The amplification reaction was carried out in a thermal cycler (Master Cycler Gradient, Eppendorf, Germany) as standardized (Ruba *et al.*, 2015), the final reaction was held at 4°C, the amplicons was analyzed by electrophoresis in 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) and images were captured using a transilluminator (Alpha imager, USA).

Detection of the subtype of AI viruses

Viral RNA from the trachea of matrix gene positive birds were used in RT-PCR amplification of HA and NA genes of AI viruses (Table 1). Published primer sequences of the HA and NA genes of AI viruses were used in RT-PCR amplification of the viral genomes (Ruba *et al.*, 2015). The cDNAs of HA and NA genes as obtained through RT-PCR were gel cleaned and sequenced from commercial source (1st Base, Malaysia). The retrieved raw sequence data were first checked for its quality and then edited. Following sequence edition a total of 1122 nucleotide sequences of HA gene and 972 nucleotide sequences for NA gene were stand for genetic analysis. The HA and NA nucleotide sequences in the GenBank database provided by the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/genbank) were downloaded and used for phylogenetic analysis. Multiple alignments were done with Clustal W algorithm and Neighbour-Joining

phylogenetic tree was constructed with MEGA6 programme. The stability of the nodes in the phylogenetic tree was tested by bootstrapping value with 1000 replications. For comparative study, available sequences of other Bangladeshi isolates of AI were downloaded from GenBank and used in phylogeny.

RESULTS

Investigation of clinical signs

A total of 20 broilers from two live bird markets of Mymensingh city were investigated. The sick birds (n=06) showed dizziness, inappetence and isolated in cages whereas the apparently healthy birds (n=12) appeared bright at the time of investigation. Two of the sick birds showed massive hemorrhages on ventral surface of the wings (Figure 1a). Four birds showed drooling of saliva through the beak, depressed and isolated in the corner of the cages. Two dead broilers were collected at time of investigation but dominant change in their external surface was not seen.

Pathological investigation

Systemic investigations were carried out onto the dead, sick and apparently healthy birds. The lungs of the sick birds were congested and consolidated (Figure 1b) and containing serofibrinous exudates onto the surface of the lungs. Lesions onto the visceral organs of apparently healthy birds were not seen at necropsy. The trachea of apparently healthy birds was almost clear and free from hemorrhages. Mild congestion was seen in the trachea of sick and dead broilers (Figure 1c).

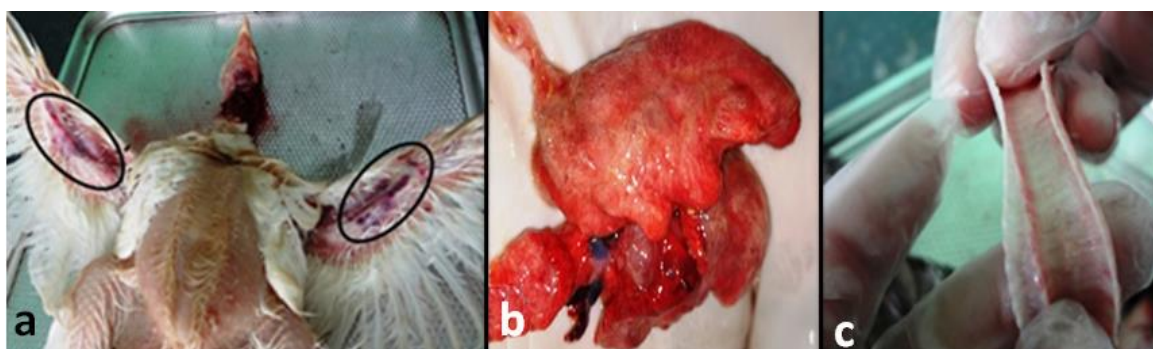


Figure 1. A broiler showed massive hemorrhages under the wing (a) at time of investigation. Following sacrificed the sick/dead birds showed congested lungs (b). Mild congestion was seen in the tracheal mucosa (c) of sick/dead broilers.

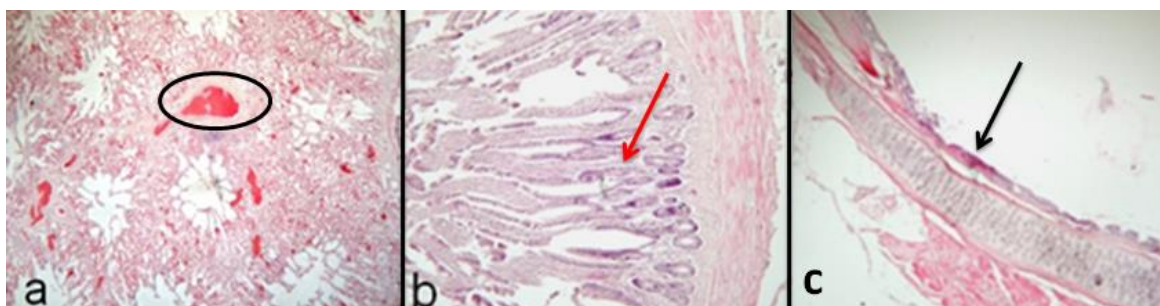


Figure 2. Histopathological examination of lungs of sick and dead birds showed wide spread congestion (a, circle), hemorrhages and interstitial pneumonia (a). The duodenum of sick birds showed mild congestion and hemorrhages (b, red arrow). There was infiltration of lymphocytes and monocytes with hemorrhages in the tracheal mucosa of dead broiler (c, black arrow).

Histopathological investigation of visceral organs

Tissue sections from the sick and apparently healthy birds were stained with H&E stain showed mild congestions and hemorrhages. The inflammatory changes were milder in the trachea and lungs of sick birds. The lungs of dead birds showed wide spread congestion, hemorrhages and interstitial pneumonia (Figure 2a). The duodenum (Figure 2b) and trachea (Figure 2c) of sick and dead birds showed congestions, hemorrhages and infiltration of mononuclear cells. Sections of the trachea of dead birds while stained with H&E showed congestion (c, arrow), hemorrhages and inflammations at various degrees (Figure 2c).

RT-PCR detection of M, HA and NA genes

Out of 20 samples tested in matrix protein gene specific RT-PCR, 430bp amplicon was generated in 07 cases (Figure 3). Type A influenza viral infectivity was higher at Kewatkhali live bird market (40%) compared to a low rate of infectivity (30%) in the broilers of BAU live bird market.

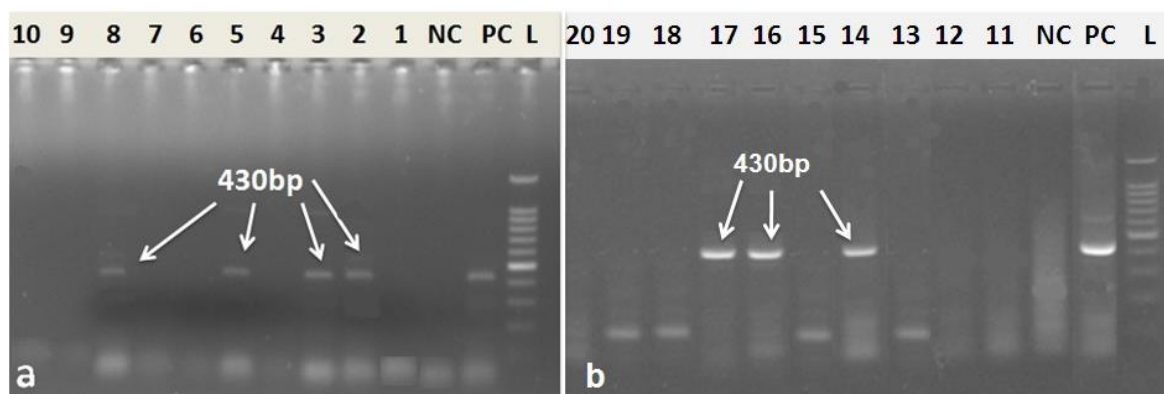


Figure 3. RT-PCR detection of M protein gene of Type A influenza virus from the broiler of live bird market of Kewatkhali (a) and BAU (b), Mymensingh. Out of 20 tracheal samples (RNA) investigated in RT-PCR, 04 samples from Kewatkhali market (a) and 03 samples (b) from BAU live bird market found to generate 430bp amplicon specific for infectivity due to Type A influenza virus. The lane L is for 100bp ladder, PC is for positive control, NC is for negative control and lane 1-20 are for test samples.

Viral RNA from the M protein gene specific samples while used in RT-PCR detection of HA and NA genes of Type A influenza virus, all 07 samples found to generate 1475bp and 1089bp amplicons (Figure 3), specific for HA and NA genes of AI virus respectively.

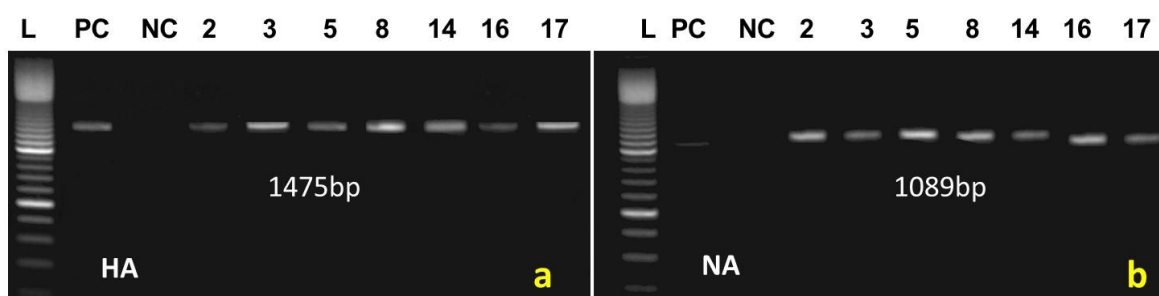


Figure 3. RT-PCR amplification of HA and NA genes of AI viruses from the matrix protein gene positive samples (Lane 2, 3, 5, 8, 14, 16 and 17). In positive cases 1475bp (a) and 1089bp (b) amplicons specific for HA and NA genes were generated, respectively. Lane L is for 100bp ladder, PC is for positive control and NC is for negative control.

Subtyping of AI viruses

Analysis and comparison of nucleotide and deduced amino acid sequences of the HA genes comprised 1122 nucleotides encoding a protein of 374 amino acids, which includes complete receptor-binding, cleavage and glycosylation sites. None of the isolates exhibited insertions or deletions within this region upon comparison with the HA gene of the H9N2 subtype prototype (A/turkey/Wisconsin/66). Numerous instances of nucleotide substitution point mutations within the gene were observed (data analysis in progress). Analysis of percent identity and divergence among nucleotide sequences of the HA genes of some other H9N2 isolates showed that the virus was fallen in H9 numbering prototype strain. Analysis of the NA gene of Type A influenza virus comprises 972 nucleotides encoding a protein of 324 amino acids which include hemadsorbing sites (HBS, 366–373, 399–403 and 431–433, H9 numbering), active center (140–157) and antigenic determinants (153, 197–199, 328–336, 339–347, 367–370, 400–403 and 431–434). The genetic and phylogenetic analysis of NA gene revealed that the AI virus is belonging to branch I strains (had a mutation at position 369, D369G) and was N2 subtype. The virus investigated was H9N2 subtype.

DISCUSSION

Influenza is the cause of seasonal respiratory illness in mans and animals which was previously thought to be species specific. Among influenza viral types, Type A virus is highly mutagenic and causes infection in a variety of host species (Righetto and Filippini, 2018; Suzuki and Nei, 2002). Influenza affects variety of hosts but avian species were the original, natural host for influenza viruses (Suarez and Schultz-Cherry, 2000) and is highly susceptible for Type A influenza viruses. It is accepted that continued contact between avian influenza viruses and mammals has allowed certain subtypes of the virus to establish itself in non-avian species like swine, horses and humans (Perdue and Suarez, 2000).

Avian influenza is popularly known as “fowl plague” and has remarkable significance due to high rate of morbidity and mortality of infected chickens (Swayne, 2008). Human and poultry populations had been plagued by “Spanish flu” during 1918 and 1919 and since then a strong association was established between avian influenza and mammalian influenza A viruses (Horimoto and Kawaoka, 2001). Globally outbreaks of influenza have devastating economic impacts on trade of poultry, poultry meat, eggs and infectivity in human and animal. Approximately 250,000-500,000 global people were died each year due to complicated association of Type A influenza viruses during the course of illness (Katsurada *et al.*, 2017; WHO, 2003). For the devastating outstretch and economic significance, this work was attempted to address the pathology and diagnostic attempts related to the infectivity of broilers in the live bird market with Type A influenza virus.

In this study, two dead, six sick and 12 apparently healthy birds were investigated by clinicopathological investigation. Following necropsy, non-specific mild hemorrhages in trachea and congestions in lungs were seen. Out of 20 tracheas examined at necropsy, mild congestion in the mucosa was seen in two cases. A bird died due to nonspecific cause showed mild congestion and consolidation of lungs and mild hemorrhages in the trachea. Characteristics cyanotic lesions in wattle, comb and shank (Bari *et al.*, 2009) was not seen in any birds. By clinical and pathological investigation, specific cause of illness (N=06) and death (N=02) of broilers was unable to identify.

For histopathological investigations, sections of lungs, liver, kidney, spleen, heart, pancreas, intestine, gizzard and skin from the sick, dead and apparently healthy broilers were stained with H&E stain. Tissue sections of dead broilers showed wide spread inflammation in lungs but was not indicative for specific disease. Infiltration of reactive cells was seen in interstitium of lungs with variable rate of congestions and hemorrhages. Birds infected with influenza virus causes massive congestion and hemorrhages in lungs with exudation in lungs alveoli (Bari *et al.*, 2009; Ruba *et al.*, 2015), such lesion was absent in broilers. Moderate congestion in heart was also seen in the dead birds. Specific microscopic alteration was not seen in any organs investigated. By histopathological investigation of visceral organs of broilers, specific cause of illness and death was not suspected. Tracheal tissues (N=20) were, therefore, used in viral RNA extraction and RT-PCR detection of M protein gene of Type A influenza virus.

The AI virus containing 08 segments of negative-sense (complementary to the mRNA) single-stranded RNA (Cattoli *et al.*, 2009) which were encoded eleven viral proteins. In this study, matrix protein gene was selected to amplify and detect fragment of M protein gene (430bp) in 07 cases with the RNA extracted from the trachea of broilers. A known positive control was included in the RT-PCR setting and in the gel electrophoresis. The broilers (about 35%) in the live bird market found to carry Type A influenza virus in their trachea. Two sick, one dead and four apparently healthy broilers carrying Type A influenza virus in their upper respiratory tract. The sick birds and apparently healthy birds although contacting Type A influenza virus but did not show any sign and lesion suggestive to infectivity due to Avian influenza virus. Two more RT-PCR protocols were, therefore, carried out with the RNA positive to M protein gene of Type A influenza virus to identify the subtype of AI virus involved. All the seven samples found to generate HA and NA gene specific amplicons in RT-PCR. The cDNAs of HA and NA genes were sequenced, sequence analyzed and phylogenetic analysis was carried out; the circulating virus was fallen into H9N2 subtype. The epidemiological dynamics, biological characteristics and molecular phylogeny of the H9N2 viruses is yet to carry out (study is in progress).

H9N2 subtype avian influenza viral outbreaks were firstly reported in Guangdong province, China in 1992. Subsequently, the disease spreads into vast majority regions in China and become endemic (Gu *et al.*, 2017). AI viral subtype H9N2 is now endemic globally in poultry producing ecology; small proportion of poultry-exposed humans has contracted the virus and the disease is often mild in nature. Globally the H9N2 virus infect only a small number of humans, because of its milder pathogenicity and widespread global distribution in poultry with poor immune response against the virus (Khan *et al.*, 2015). Over vicennial genetic evolution, the viral pathogenicity and transmissibility of H9N2 AI viruses have showed an increasing trend to infect poultry industry. The H9N2 virus has also demonstrated its great significance to public health because it directly infect mankind and also donate partial or even whole cassette of internal genes to generate novel human-lethal reassortants like H5N1, H7N9, H10N8 and H5N6 viruses (Gu *et al.*, 2017).

Occasionally, human cases were identified through active influenza surveillance in human in Bangladesh during 2011–2015; the cases reported would have exposure to poultry or poultry products (e.g. raw meat) purchased from urban live bird markets (ICDDR'B 2011). It is assumed that many humans were infected with H9N2 viruses in Bangladesh through continuous exposure to infect poultry and live bird markets, but were never tested for influenza due to lack of consciousness, poor illness or asymptomatic infections (Azziz-Baumgartner *et al.*, 2012). This study investigated 20 respiratory tissues of broilers from selected live bird market of Mymensingh city to know the type of AI virus involved. Out of 20 birds investigated, typical signs and lesions suggestive of AI was not distinguished in any of the birds but 02 sick, 01 dead and 04 apparently healthy birds found to carry H9N2 virus.

Until now the H9N2 subtype is distinguished as low pathogenic AI but very much challenging for poultry industry. The H9N2 virus was considered as the early isolate from turkey flocks in Wisconsin in America in 1966 [A/turkey/Wisconsin/1/1966(H9N2)] and became more and more extensively at about 1990s, resulting continuous viral circulation in several countries in Asia, Middle East and North Africa (Alexander, 2007). The H9N2 AI virus may cause illness of the birds with indistinct pathology, leading favorable circumstances for co-infection, immunosuppression, morbidity and mortality (Sun and Liu, 2015). The H9N2 virus also provide threat for infecting mankind directly.

CONCLUSIONS

This study investigated 20 broilers from KR market, BAU, Mymensingh and Kewatkhalil local market, Mymensingh and 07 birds found to carry Type A AI viruses in their upper respiratory tracts. Clinical and pathological investigation was unable to trace infectivity of broilers due to AI viruses. The RT-PCR protocols used appeared successful in terms of detecting M, HA and NA genes of Type A influenza virus and identify H9N2 viruses in the dead, sick and apparently healthy broilers. The occurrence of H9N2 AI viruses in the broilers of live bird market possess a substantial threat to public health and the carrier birds may prone to co-infection with other infectious agents having affinity to upper respiratory tract. It requires extensive surveillance on to the distribution of H9N2 AI virus and other subtypes, identifying the effect of mutation on receptor binding domains (study in progress) and predicting possible generation of pandemic AI viruses.

CONFLICT OF INTEREST

The authors have no affiliations with or involvement in any organization or entity with any financial and non-financial interest in the subject matter or materials stated in this manuscript.

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