Investigation of an Outbreak of Low Pathogenic Avian Influenza in Poultry in Bangladesh

Jannat, N. 1, Chowdhury, E. H. *1, Parvin, R. 1, Begum, J.A. 1, Giasuddin M. 2, Khan, M. A. H. N. A. 1 and Islam, M. R. 1

1Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh
2Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka, Bangladesh.

*Corresponding Author: emdad001@yahoo.com

Rec. Date: Aug 03, 2013 22:37; Accepted Date: Sep 17, 2013 08:38

Abstract

Since March 2007, an epidemic of highly pathogenic avian influenza has been devastating in backyard and commercial poultry in Bangladesh. The agents were pathotyped as highly pathogenic H5N1. This investigation reports the result of a retrospective investigation of an outbreak of low pathogenic H9N2 avian influenza in commercial parent stock chickens that occurred in 2006. The morbidity and mortality were 50% and 3%, respectively during 2–3 months. The egg production decreased to 36% and hatchability reduced to 10%. The clinical signs included less feed and water intake, nasal and ocular discharges and severe respiratory distress. On postmortem, samples including larynx, trachea and lungs were collected in 10% neutral buffered formalin and tracheal swabs, larynx and tracheal tissues were collected in Falcon tube containing 50% buffered glycerin with antibiotic and stored frozen at -70 °C. Histopathology was conducted using routine procedure. RNA extraction and RT-PCR were done using Qiagen RNA extraction and one step RT-PCR kits. Grossly, hemorrhage in nasal septum, sinuses, eyelids, larynx and trachea were found. Lungs were congested. Histologically, in larynx and trachea loss of mucosal epithelium, hemorrhages and mononuclear cells infiltration in lamina propria, blood tinged exudates in laryngeal and tracheal lumen were observed. In few cases, goblet cells were hypertrophied and mucosal glands were prominent that formed cystic spaces. RT-PCR confirmed the presence of 245 bp product of matrix protein gene and 488 bp fragment of H9 subtype specific haemagglutinin gene as well as 244 bp fragments of N2 gene in the suspected samples. The study proved that H9N2 low pathogenic avian influenza virus was introduced in Bangladesh before the detection of H5N1 viruses.

Keywords: Low pathogenic, avian influenza, RT-PCR

Introduction

Avian influenza is an infectious disease of birds caused by influenza virus type A strains. It belongs to the Orthomyxoviridae family (Lamb, 1989; Lamb and Krug, 1996). Orthomyxoviruses are spherical or pleomorphic, enveloped and 80-120 nm in diameter (Quinn et al., 2002). This is an RNA virus having the negative-sense segmented ssRNA genome having
eight segments. The virus envelope is a host-derived lipid bilayer (Quinn et al., 2002) and covered with about 600 projecting glycoprotein spikes with hemagglutinating and neuraminidase activities. Wild aquatic birds, notably members of the orders Anseriformes (ducks and geese) and Charadriiformes (gulls and shorebirds), are carriers of the full variety of influenza virus A subtypes, and thus, most probably constitute the natural reservoir of all influenza A viruses (Webster et al., 1992; Fouchier et al., 2003; Krauss et al., 2004; Widjaja et al., 2004). While all bird species are thought to be susceptible, some domestic poultry species - chickens, turkey, guinea fowl, quail and pheasants are known to be especially vulnerable to the sequelae of infection.

While high pathogenic avian influenza (HPAI) viruses of H5N1 subtype have received great attention because of devastating damage to the poultry industry and occasional human infection, low pathogenic avian influenza (LPAI) viruses of subtype H9N2 are also widespread in poultry (Peiris et al., 1999). H9N2 influenza viruses are endemic in many Asian countries (Kim et al., 2006, Alexander, 2007). Infections of domestic avian species with LPAI viruses can be asymptomatic or cause a wide range of clinical signs varying from mild respiratory disease to more severe diseases affecting the respiratory and enteric systems. Bangladesh encountered the first outbreak of HPAI (H5N1) on 5th February, 2007 and thereafter the virus has spread in many parts of the country in commercial and backyard chickens. The present paper reports an outbreak of LPAI in a parent stock that occurred in 2006 but confirmed through retrospective analysis of laboratory samples after the emergence of HPAI in Bangladesh in 2007.

**Materials and Methods**

**Clinical investigation and collection of samples**

This study involves the investigation of an outbreak of mild respiratory disease in a commercial parent stock of chickens in 2006. The history of the outbreak and clinical signs were recorded from the veterinarian of the farm. Routine necropsy was done on 2 dead birds and lesions were recorded. Tissue samples from larynx, trachea and lungs were collected and preserved in 10% neutral buffered formalin for histopathology. Pieces of tracheal tissues were also collected in 50% buffered glycerol and stored frozen at -70 °C aseptically for isolation and molecular detection of virus.
Histopathology

Formalin-fixed tissues were processed for paraffin embedding, sectioned and stained with haematoxylin and eosin as per standard procedure (Luna, 1968). Stained sections were examined under low and high power objectives of a microscope.

Reverse transcription polymerase chain reaction (RT-PCR)

Based on necropsy, the case was first tentatively diagnosed as infectious laryngotracheitis (ILT) and accordingly tested for ILT virus by PCR. After the emergence of avian influenza in Bangladesh, the sample was tested retrospectively for Type A avian influenza virus matrix protein (M) gene, H5 and H9 haemagglutinin (HA) gene and N2 neuraminidase (NA) gene. Tracheal tissue samples were homogenized aseptically with mortar and pestle to prepare 20% (w/v) homogenate in PBS. Total RNA was isolated from the tissue homogenate using Qiagen RNeasy kit following the manufacturer’s instructions. One-step RT-PCR was done with Qiagen RT-PCR kit using primers and thermal profiles mentioned in Table 1. RT-PCR products were analyzed by electrophoresis on 1.5% agarose gel, stained with ethidium bromide and examined against UV light using an image documentation system.

Table 1 Primers and thermal profile used in RT-PCR for avian influenza viruses investigation

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Target virus &amp; gene</th>
<th>Thermal profile</th>
<th>Expected Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILTV PCR5’</td>
<td>5’-ACGATGACTCCGACTTTTC-3’</td>
<td>TK gene of ILTV</td>
<td>35 cycles of Denaturation 95°C for 1 min Anneling 50°C for 1 min Elongation 72°C for 1.5 min</td>
<td>647 bp</td>
<td>Griffin et al. (1990)</td>
</tr>
<tr>
<td>ILTV PCR3’</td>
<td>5’-CGTTGGAGGTAGGTGGTGTA-3’</td>
<td>MP</td>
<td>RT at 50°C for 30 min 40 cycles</td>
<td></td>
<td>Fouchier</td>
</tr>
<tr>
<td>M253 (Reverse)</td>
<td>5’-AGG GCA TTT TGG ACA AAK CGT CTA-3’</td>
<td>gene of AIV of PCR</td>
<td>245 bp</td>
<td><em>et al.</em> (2000)</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------------------</td>
<td>---------------------</td>
<td>--------</td>
<td>----------------</td>
<td></td>
</tr>
<tr>
<td>H5-155F</td>
<td>5’-ACACATGCYCARGACATACT-3’</td>
<td>H5 gene of HPAIV RT at 50°C</td>
<td>545 bp</td>
<td><em>Lee et al.</em> (2001)</td>
<td></td>
</tr>
<tr>
<td>H5-699R</td>
<td>5’-CTYTGRTTYAGTGTGTTGATG-3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9-151F</td>
<td>5’-CTY CAC ACA G A CAC AAT GG-3’</td>
<td>H9 gene of LPAIV RT at 50°C</td>
<td>488 bp</td>
<td><em>Lee et al.</em> (2001)</td>
<td></td>
</tr>
<tr>
<td>H9-638R</td>
<td>5’-GTC ACA CTT GTT GTT GTR TC-3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2-39f</td>
<td>5’-TCT CTC TAA CCA TTR CAA CAG TAT G-3’</td>
<td>N2 gene of LPAIV RT at 50°C</td>
<td>244 bp</td>
<td>[NIAH, Bangkok]</td>
<td></td>
</tr>
<tr>
<td>N2-283r</td>
<td>5’-TAA TTT GAC ATT GYG GCT TTG ACC A-3’</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Annealing 50°C, 90 sec
Elongation 68°C, 2 min

*Inclusions of degenerate nucleotides are indicated in bold. Codes for mixed bases position:

\[
R = A/G, \quad M = G/C, \quad K = G/T, \quad Y = C/T, \quad H = A/C/T, \quad B = G/C/T
\]

**Embryo inoculation and haemagglutination test**

The tissue homogenates were treated with gentamycin (500 μg/ml) and inoculated in 10-day old embryonated chicken eggs @ 200 μl/egg through allantoic cavity route using 5 eggs per sample. After death of embryos or 3 days after inoculation the eggs were chilled and allantoic fluid was aspirated aseptically. The allantoic fluid was tested for haemagglutinating activity by microtitre plate haemagglutination test (OIE, 2000).

**Results and Discussion**

**History and clinical signs**

An outbreak of respiratory infection in a layer parent stock farm of Mymensingh district was investigated. The outbreak started in September 2006. The birds were aged between 42–65 weeks. Birds had been vaccinated against infectious bursal disease, Newcastle disease, infectious bronchitis and reovirus.

Over a period of two months the morbidity and mortality were 50% and 3%, respectively. The egg production decreased to 36% and hatchability decreased to 10%. Mild rales and gasping as well as reduced feed and water intake were observed in infected chickens. these findings were similar to that observed by others (Nili and Asasi, 2002, 2003; Elbers et al., 2005; Kim et. al., 2006; Trani et al., 2006; Kwon et al., 2006, 2006a) for low pathogenic avian influenza virus.

**Gross and histopathology**
At necropsy haemorrhagic lesions were found in the larynx and trachea (Figure 1). Lungs were congested. Peritoneum, air sac and pericardium were thickened and cloudy.

Histologically, in the larynx and trachea, there were losses of mucosal epithelium, accumulation of exudates containing desquamated, degenerated and necrotic epithelial cells. Presence of hemorrhage in the lumen of the trachea, and congested blood vessels along with hemorrhages and mononuclear cells infiltration in lamina propria (Figure 2). Increased numbers of goblet cells were seen in some cases. Similar findings were also observed by different authors (Nili and Asasi, 2002; Nili and Asasi, 2003; Kwon et al., 2006; Nili et al., 2007).
Figure 2 Section of larynx and trachea of a naturally infected chicken showing A) loss of mucosal epithelium (H & E, ×82.5). B) desquamated epithelial cells and red blood cells in the lumen (H & E, × 82.5). C) hemorrhagic lamina propria with loss of its covering (H & E, × 82.5). D) hemorrhages and infiltration of mononuclear cells in lamina propria (H & E, × 82.5). E) inflammatory cells around the blood vessels in submucosa (H & E, ×82.5). F) hypertrophied goblet cells and prominent mucosal glands forming cystic spaces (H & E, ×82.5)

**Virus isolation and identification**

**Embryo inoculation**

After inoculation into embryonated chicken eggs through allantoic route the virus isolates caused embryo mortality, and the rate of embryo mortality increased with the passage (Table 2). In the first passage only 40% embryo died at 72 hours after inoculation. However, on the second and third passage the mortality reached up to 90% and most of the death occurred within 48 hours after inoculation. No embryo mortality was observed in uninoculated controls.
Table 2. Embryo mortality following inoculation with the virus isolate

<table>
<thead>
<tr>
<th>Passage</th>
<th>Number of embryos died</th>
<th>Number of embryos surviving at 72 hrs</th>
<th>Cumulative mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hr</td>
<td>48 hr</td>
<td>72 hr</td>
</tr>
<tr>
<td>First passage (n=10)</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Second passage (n=9)</td>
<td>0</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Third passage (n=10)</td>
<td>0</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>

Haemagglutination (HA) test

The haemagglutination (HA) test was performed to determine the presence of haemaglutinating agent in the allantoic fluid. Results indicated that the allantoic fluid had haemagglutination activity.

RT-PCR for ILTV

The samples were tested with RT-PCR for TK gene of infectious laryngotracheitis virus (ILTV) and found negative.

RT-PCR for matrix protein (M) gene

The samples were tested with RT-PCR for M gene and found positive and yielded a product of about 245 base pair (bp), as expected (Figure 3)
Figure 3 Amplification of the fragment of matrix protein gene of Type A influenza virus by RT-PCR. M=Marker, S1=Field sample-1, S2= Field sample-2, PC= Positive control (RNA extracted from inactivated H9 virus received from VLA, England).

RT-PCR for haemagglutinin (H5 and H9) gene and N2 gene

The samples were then tested for H5 and H9 subtype-specific haemagglutinin genes using primers designed by Lee et al. (2001). The RT-PCR for H9 haemagglutinin gene was positive as it amplified a product of about 488 bp from the samples (Figure 4). However, RT-PCR for H5 haemagglutinin gene using primers designed by Lee et al. (2001) did not amplify any product. Several modifications in the time and temperature for annealing stage did not make any difference. Amplification of N2 gene was also carried out successfully by using N2 gene specific primer (NIAH Bankok) as it amplified about 244 bp from the samples (Figure 5).

Figure 4 Amplification of the fragment of H9 gene by RT-PCR. M=Marker, NC=Negative control  S1P3=Field sample-1Passage 3, S2P3= Field sample-2 Passage 3, S1P2= Field Sample -1 Passage 2, S2P2= Field sample 2 Passage 2, S1P1= Field Sample-1 Passage 1, S2P1= Field Sample-2 Passage 1.
Figure 5 Amplification of the fragment of N2 gene by RT-PCR. M=Marker, NC=Negative control, W= Water control, P1=Passage-1, P2= Passage -2 and P3= Passage-3.

This study confirms LPAI infection in a layer parent stock farms in Bangladesh much before the incursion of HPAI outbreak in Bangladesh. Subsequently further outbreak of H9N2 virus has been reported in the poultry farm in Bangladesh either as single or concurrent infection with H5N1. The clinical signs, gross pathological and histopathological findings in natural H9N2 LPAI infection in chickens often could be confused with other respiratory viral infections, hence would need confirmation by molecular techniques.

Conclusion

Low pathogenic avian influenza (H9N2) virus has been circulating in poultry farms of Bangladesh since 2006.

Acknowledgements

Authors’ are thankful to the Third world Academy of Science (TWAS) for research funding.

References


18. Trani LD, Bedini B, Donatelli I, Campitelli L., Chiapini B, Macro M, Delogu MAD, Bonavoglia C and Vaccari G. 2006. A sensitive one- step real-time PCR for detection of
avian influenza viruses using a MGB probe and an internal positive control. *BMC Infectious Diseases* 6:87.
