

CO-CIRCULATION OF THREE SUB-TYPES OF AVIAN INFLUENZA VIRUSES IN THE POULTRY POPULATION OF BANGLADESH IN THE YEAR 2014

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ABSTRACT

A research work was undertaken to detect circulating sub-types of avian influenza viruses in five different species of poultry (chicken, duck, goose, pigeon and quail) in Bangladesh during January to September 2014. A total of 210 cloacal swab samples were collected for AIV by rapid test kit (RTK), hemagglutination inhibition test (HIT), neuraminidase inhibition test (NIT) and RT-PCR. Of the total 210 samples, 75 (35.71%) were positive for AIV and the remaining 135 (64.28%) were negative by RTK. Out of 75 positive samples by RTK, 60 (80%) from chickens, 10 (13.33%) from ducks and 5 (6.66%) from goose. None of the samples from pigeon and quail found positive for AIV by RTK. The RTK positive samples were subjected to the HIT and NIT using monospecific panel of serum against AIV (H1-15 and N1-9). Of the 75 positive samples 53 were H5N1, 18 were H9N2 and 4 were H7N9 positive by HIT and NIT. Out of the 75 serologically positive samples, 60 (80%) from chickens, 10 (13.33%) from ducks and 5 (6.66%) from goose. On the contrary, RT-PCR results revealed 80 (38.09%) samples were positive samples for AIV out of 210 samples. Of the 80 AIV positive samples, 55 were H5N1, 20 H9N2 and 5 H7N9. Out of 80 RT-PCR positive AIV samples 65 (72.22%) from chickens, 10 (13.33%) from ducks and 5 (6.66%) from goose. Findings of the study indicated that out of three sub-types of AIV H5N1 was predominant compared to that of other two sub-types. Overall findings of this study clearly indicated that the three sub-types of AIV along with H7N9 in Bangladesh.

KEYWORDS: AIV, RTK, HIT, NIT and RT-PCR

INTRODUCTION

Avian influenza (AI) is an infectious disease of domestic, semi- domestic and wild birds ranging from a mild to severe form of illness which is caused by avian influenza virus (AIV), a single stranded negative-sense enveloped RNA virus belonging to the family *Orthomyxoviridae* (Davison *et al.* 1998). There are three types of influenza viruses namely A, B, and C where viral genome is comprised of eight RNA segments (seven in type C). Among these types, type A infects a wide range of animal's species such as humans, pigs, horses, marine mammals and birds (Nicholson *et al.* 2003) while aquatic birds are the major reservoir of these viruses (Hinshaw *et al.* 1981). Influenza A viruses infecting poultry can be divided into two distinct groups on the basis of their ability to cause disease named highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) viruses (Alexander 2000a). Influenza A viruses can be classified into sub-types based on antigenic differences in two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) which are required for viral attachment and cellular release (Chan 2002). Other major viral proteins include the nucleoprotein (NP), which is the main structural protein, membrane proteins (M1 and M2), polymerase proteins (PA, PB1 and PB2), and non-structural proteins (NS1 and NS2). Currently, sixteen sub-types of HA (H1-H16) and nine NA (N1-N9) antigenic variants of influenza A virus have been reported all over the world which are mostly related to veterinary significance. Avian influenza is a "notifiable" highly infectious disease affecting many species of birds, including chickens, duck, turkeys and geese (Munster *et al.* 2005).

It can affect commercial and pet birds as well. There are various sub-types of bird flu, but the sub-type that is concerned at the moment is the deadly sub-type H5N1. AIV do not replicate well in mammals indicating birds to human

transmission is unlikely to occur. However, since 1997 several cases of H5N1 and H7N9 in human raised concern in health veterinary professions.

MATERIALS AND METHODS

Cloacal swab samples were collected from sick and dead farm birds of Bangladesh namely commercial broiler, layer, native birds, duck, goose, pigeon and quail. The samples were collected and stored at 4°C in the ice box maintaining cool chain. For initial screening, the tests were performed with the help of RTK (Anigen Rapid AIV test kit, Korea). Results of the tests were observed within 3-5 minutes recorded by naked eye detection of single band for negative control, double bands for the AIV Ag. The presence of two bands on the AIV Ag test area indicates positive result of avian influenza virus type A. Then the hemagglutination Inhibition (HI) test was also performed using all samples. For sub-typing of AIV, further more specific quantitative sub-type specific reference antisera against each of the 15 sub-types (H1-15/N1-9) of AIV done according to the instruction of OIE reference laboratory of Hokkaido University, Japan. Finally, all the samples those were found positive and negative for AIV in RTK and HI test were further confirmed by reverse transcription polymerase chain reaction (RT-PCR) using gene specific primers against different sub-types of AI viruses after extraction of viral mRNA from cloacal swab samples.

Haemagglutination (HA) test

Micro haemagglutination test was performed in a V-bottom 96 micro-well plate to determine HA units (4HA/50µl). This was carried out by two-fold serial dilutions of the viral suspension in a micro-well plate and then tested to determine an end point. For this purpose, 50 µl of PBS was dispensed into each well of the micro-well plate. Then 50 µl of test sample (virus) were placed in first well of each row of column 1 and then two-fold dilution was made up to column 11. A 100 µl of 1 percent chicken red blood cells were added to each well including wells of column 12. The control wells contain only PBS and red blood cells. The plate was allowed to stand for 45 minutes in the refrigerator at 4°C.

The results of the plates were read and recorded according to Reed and Muench (1938) method. In HA negative case, a sharp buttoning of red blood cells at the bottom of the V-bottom well and in HA positive case, button of red blood cells at the bottom of the V-bottom wells were showed in micro well plate. The HA of the virus was determined as the highest dilution of the virus which agglutinates the RBC in the test. 4HA of the virus was calculated from the 1HA.

Haemagglutination Inhibition (HI) test

The HI test was performed to determine the inhibitory activity of monospecific antibody against each of the fifteen sub-types for each of the HA positive cloacal swab samples collected from the chickens (broiler, layer and native), duck, goose, pigeon and quail. The test was conducted by using constant 4 HA unit antigen and increasing serum dilution method (B procedure) following the procedure described by Reed and Muench (1938). The test was done as per the method described in OIE manual (2004).

Neuraminidase assay (NA)

Serial dilution of field isolates of AIV prepared in PBS (pH 7.3) usually in $0.5 \log^{10}$ (1/3.16) serially 10^0 , $10^{-0.5}$, 10^{-1} , 10^{-2} , $10^{-2.5}$, 10^{-3} . The $10^{-0.5}$ dilution means 0.1 µl of undiluted virus + 0.216 µl of PBS. Duplicate tubes for each dilution were prepared in the order to assay

- Virus sample: 50 µl of each virus dilution + 50 µl of PBS + 100 µl of fetuin;
- negative control: 100 µl of PBS + 100 µl of fetuin;

The optical density (OD) was measured by spectrophotometer at wavelength 549 nm. The proper virus dilution was determined to be used in the neuraminidase inhibition assay by constructing a NA curve for each field sample tested.

Neuraminidase inhibition assay (NIA)

A total of 10 tubes including one negative control were used. The antiserum of 10 µl added to tubes marked with respective NA (N1-9) sub-types. 10 µl of each virus added to the each of 9 tubes. 10 µl of PBS was also added to each tube. The tubes were shaken well, covered and incubated at 37°C for 1 hr. Fetuin substrate of 20 µl added to all tubes and shaken well and finally incubated 37°C for overnight. Then periodate reagent of 20 µl added to all tubes and shaken well and left for 20 minutes at room temperature. Then 200 µl of arsenite reagent was added to each tube and the brown color was appeared in each tube and all the tubes were shaken vigorously until the brown color disappeared. Then 500 µl of thiobarbituric acid added to each tube and shaken vigorously and then the tubes were boiled in water. The tubes were then cooled on ice and 800 µl of warren off reagent was added. Finally the tubes were vortexed for 10 sec and centrifuged at 2000 rpm for 10 minutes. The upper phase of the fluid was then transferred to a cuvette and the OD value was measured by 549 nm.

RNA extraction:

RNA extraction was done in the class-100 laminar airflow under BSL-3 laboratory of the R&D section of FnF Pharmaceutical and Biological Company Limited. The genomic viral RNA from AIV was extracted using 140.0 µl of clinical (cloacal swab) samples using the QIAamp mini RNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. A volume of 4 µl of the eluted RNA of AIV and 8.3 µl of DEPC were taken into individual PCR tube (DNase and RNase free) and mixed properly with the help of the minispin. The tubes were then placed in a forty eight wells thermocycler (Mastercycler, Eppendorf, Hamburg, Germany) and applied the thermal profile as 94°C temperatures for 5 minutes for linearization of coiled RNA followed by snap cooling on ice for 2 minutes to stay linearized. Meanwhile, for the synthesis of cDNA from RNA of AIV reaction mixture containing 5XRT buffer 4.0 µl, 10 mM dNTP 2.0 µl, prime RNase inhibitor 1.0 µl, AMV-RT 0.2 µl, primer (RH 100 pmol) 0.5 µl was prepared and kept on ice. After adding this reaction mixture onto the PCR tube containing linearized RNA of AIV placed into the thermocycler and followed the thermal profile of 42°C for 60 minutes followed by 85°C for 5 minutes. For the synthesis of DNA from cDNA of HA (H5N1, H9N2 and H7N9) of AIV, reaction mixture were used as 50 µl volume comprising 10X LA buffer 5.0 µl, 25 mM MgCl₂ 2.0 µl, 10 mM dNTP 2.0 µl, LA-Taq 0.2 µl, specific primers (**Table 1**) H5F (100 pmol) 0.3 µl, H5R (100 pmol) 0.3 µl, H7F (100 pmol) 0.3µl, H7R (100 pmol) 0.3 µl, HAF1(100 pmol) 0.3 µl, HAR2 (100 pmol) 0.3 µl, cDNA 1.5 µl and DEPC 38.7 µl for AIV was added to each tube and mixed with the micropipette and minispin. The tubes were immediately placed to the thermocycler and the cycling programmed was resumed as : 94°C for 2 minutes, 35 cycles were continued at 94°C for 30 seconds for denaturation, 45°C for 1 min for annealing, 60°C for 1 minute for elongation and final elongation at 60°C for 10 minutes. Each PCR product was detected by 2% NA Agarose gel electrophoresis.

Table 1: List of primers used for AIV genome detection

Primers	Sequence (5' - 3')	Nucleotide position	Amplicon Size (bp)	Reference
H5-1/F (HA)	GCCATTCCACAACATMCACCC	943-963	219	Lee <i>et al.</i> (2001)
H5-3/R (HA)	CTCCCCTGCTCRRTTGCTATG	1142-1161		
HAF1(H9N2)	GCATACATCATCCACCCAC	595- 1024	430	Karimi <i>et al.</i> (2004)
HAR1(H9N2)	TTGATCTAGCAGGCACGTTC			
H7N9 Forward	ATAGATAGCAGGGCAGTTGG	916-935	672	Wong <i>et al.</i> (2013)
H7N9 Reverse	GATCAATTGCCGATTGAGTG	1137-1156		

RESULTS

Results of RTK

A total of 210 cloacal swab samples were subjected for RTK for the presence of AIV. Out of 210 samples, 75 (35.71%) were positive for AIV and the remaining 135 (64.28%) were negative by RTK. Total of 90 cloacal swab samples were collected from chicken (broiler, layer and native) different districts of Bangladesh. All the 90 samples were subjected for RTK for the presence of AIV. Among the 90 samples, 60 (66.66%) samples were positive for AIV and remaining 30 (33.33%) samples were negative for AIV. A total of 30 cloacal swab samples were collected from ducks and all the samples were subjected for RTK for the presence of AIV. Among the 30 samples, 10 (33.33%) samples were positive for AIV and remaining 20 (66.66%) samples were negative for AIV. A total of 30 cloacal swab samples were collected from goose all the samples were subjected for RTK for the presence of AIV. Among the 30 samples, 5 (16.66%) samples were positive for AIV and remaining 25 (83.33%) samples were negative for AIV. A total of each 30 cloacal swab samples were collected from pigeon and quail all the samples were subjected for RTK for the presence of AIV. Among these samples, all samples were negative for AIV.

Results of HI test

All of the 210 cloacal swabs samples suspension those were positive and negative for AIV by RTK were subjected to HI test using sub-type specific panel of serum raised against 15 sub-types of AIV. Before performing HI test, HA titration of each of the field sample was done to determine 4 HA unit. In HI test, 75 (35.71%) samples were positive for AIV, and remaining 135 (64.28%) samples were negative for AIV.

Of the 75 AIV positive samples, HA activity of 53 samples were completely inhibited by reference antisera A/tern/So.Af./61 against (H5) of AIV, 18 samples were completely inhibited by the reference anti-sera A/turkey/W1/66 (H9) *i.e.* they were positive for H9N2 of AIV and 4 samples were completely inhibited by the reference anti-sera A/equine/prague/56 (H7) *i.e.* they were positive for H7N9 and the remaining samples were not inhibited either by anti-AIV.

Results of Neuraminidase assay (NA)

A dilution of virus of 1:650 ($10^{-2.81}$) showed one unit of NA activity per 50 μ l of virus samples used in the assay. The tubes containing virus sample + anti-N1 serum A/swine/Iowa/30 serum, anti-N2 serum A/turkey/Wisconsin/66 and anti-N9 serum A/turkey/Ontario/7732/66 showed pink color indicating the positivity of neuraminidase assay.

Results of Neuraminidase inhibition assay

All the tubes assayed for NI for virus identification showed pale yellow by the disappearance of pink color of neuraminidase assay (NA). Each tube with AIV sample assayed, containing the virus sample plus reference antiserum was compared to the tube containing virus sample plus PBS (no antiserum).

In case of the tube containing virus sample + anti-N1 serum A/swine/Iowa/30 serum, virus sample + anti-N2 serum A/turkey/Wisconsin/66 and virus sample + anti-N9 serum A/turkey/Ontario/7732/66 showed pale yellow color which indicates that the NA activity was inhibited by the reference antiserum of “anti-N1 serum A/swine/Iowa/30 serum, anti-N2 serum A/turkey/Wisconsin/66 and anti-N9 serum A/turkey/Ontario/7732/66” NA sub-types. The results of NI assay clearly indicated that out of 75 positive NA samples, 53 were positive for N1, 18 for N2 and 5 for N9 sub-types of AIV.

Results of RT-PCR

All of the 210 field samples were subjected to molecular detection for the confirmation of AIV by RT-PCR using type specific primer against H5N1, H9N2 and H7N9. On the other hand, RT-PCR results revealed that of the 210 samples, 80 samples were positive for AIV. The result of RT-PCR indicated that 55 samples were found positive for H5N1, 20 for H9N2 and 5 positive for H7N9. RT-PCR products of H5N1, H9N2 and H7N9 were found to show specific bands on 219bp, 430bp and 672bp respectively, on 2% NA Agarose gel electrophoresis (**Figure 1**).

PCR image:

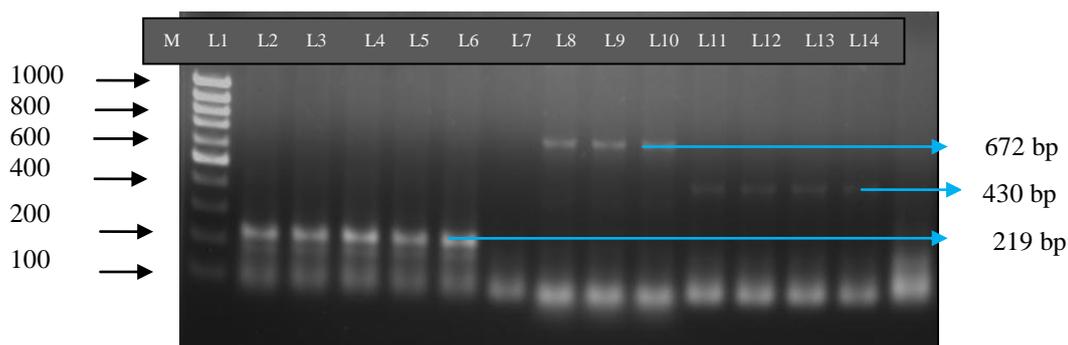


Figure 1: The RT-PCR results of cloacal swabs samples. M =100 bp DNA Marker; Lane 1= Positive control (H5N1), Lanes 2-5=Positive samples (H5N1), Lane 6= Negative control (NC), Lane 7= Positive control (H7N9), Lanes 8-9= Positive samples (H7N9), Lane 10= Positive control (H9N2), Lanes 11-13= Positive samples (H9N2), Lane 14=Negative control (NC)

DISCUSSION

Present study was undertaken to detect the avian influenza viruses from five different species of sick and dead farm birds (chicken, duck, goose, pigeon and quail) and also for sub-type determination of circulating AIV prevalent in Bangladesh. In this study, a total of 210 cloacal swab samples were collected from five different species of farm birds during the period January to September 2014 for sub-type determination of AIV through initial screening by RTK, followed by hemagglutination inhibition test (HIT), neuraminidase inhibition test (NIT) and molecular detection method. Initial screening of all the 210 samples was performed for the presence of AIV in the cloacal swabs of farm birds by using the RTK. Result of RTK revealed that out of 210 cloacal swab samples of five different species of farm birds, 75 (35.71%) were positive for AIV and the remaining 135 (64.28%) were negative by RTK in this study. A total

of 75 positive samples by RTK, 60 (80%) were positive from chickens (30% from broiler, 40% from layer and 30% from native chicken), 10 (13.33%) from ducks and 5 (6.66%) from goose. None of the swab samples from the remaining two species *i.e.* pigeon and quail revealed positivity for AIV by RTK. Overall results of RTK also indicate that the prevalence of AIV was only confined among the three species of farm birds namely chicken, ducks and goose in this study. The swab samples collected from pigeon and quail showed negative by RTK for AIV may be due to occurrence of less incidence or non carrier stage of AIV by the two species of healthy birds during sampling period. The results of RTK of the present study similarly agree with the findings of Malek (2009).

All of the 210 swab samples those were subjected for initial screening by RTK were further confirmed by HI test using sub-type specific reference sera against AIV present in the field samples in this study. For sub-typing of AIV, mono-specific panel of serum against all the 15 sub-types (H1-15 /N1-9) of AIV were used. The results of HI test indicated that 75 (35.71%) samples were positive for AIV. Of the 75 AIV (53 for H5N1, 18 for H9N2 and 4 for H7N9) positive samples, 60 (80%) samples were positive from chickens (30% from broiler, 40% from layer and 30% from native chicken), 10 (13.33%) from ducks and 5 (6.66%) from goose. Results of sub-type determination using mono-specific panel of antiserum used in this study highly agree with the findings of Chang *et al.* (2004) and Pedersen (2008). In their study, they reported that similar results were found by HI test for the detection of avian influenza virus directly from cloacal swab samples from different species of farm birds. The swab samples collected from pigeon and quail showed negative by HI test for AIV by using mono-specific panel of antiserum against AIV.

When neuraminidase (NA) and neuraminidase inhibition (NI) assay were performed with these 75 HA positive samples using mono-specific antisera against N1-9, the NA and NI result revealed that 53 samples were positive for N1, 18 were N2 and 4 were N9 sub-types which supports the finding of Panigrahy *et al.* (1995), Gyarmati *et al.* (2008) and Pederson (2008).

All of the 210 swab samples were further subjected to genome detection by RT-PCR using sub-types (H5N1, H7N9 and H9N2) specific primers of AIV. In the molecular detection method (RT-PCR), 80 (38.09%) samples showed positivity for AIV. A total of 80 AIV (55 for H5N1, 20 for H9N2 and 5 for H7N9) positive by RT-PCR, 65 (72.22%) samples were positive from chickens (22.22% from broiler, 27.77% from layer and 22.22% from native chicken), 10 (13.33%) from ducks and 5 (6.66%) from goose. The results of RT-PCR of the present study highly agree with the findings of Lee *et al.* (2001), Zhu *et al.* (2014), Karimi *et al.* (2004) and Wong *et al.* (2013). In their study, they stated the same results were observed by using sub-type specific primers set of AIV for the detection and differentiation of the sub-types of AIV viruses from cloacal swab samples from different species of farm birds. The swab samples collected from pigeon and quail showed negative for AIV by RT-PCR. Findings of the study indicated that out of three sub-types of AIV, H5N1 was predominant in chickens compared to that of other two sub-types. Overall findings of this study clearly indicated that the three sub-types of AIV are co-circulating among the three species of farm birds in Bangladesh and introduction of a new sub-type H7N9 in the native chicken might be a future potential threat to the commercial poultry in this country.

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