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CO-CIRCULATION OF VELOGENIC AND MESOGENIC STRAINS OF NEWCASTLE DISEASE VIRUS IN THE FARM BIRDS OF BANGLADESH

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ABSTRACT

A research work was undertaken to detect circulating pathotypes of Newcastle disease viruses in the farm birds (chicken, pigeon and quail) in Bangladesh during the period January to September 2014. A total of 150 cloacal swab samples were collected for the isolation and detection of NDV from dead birds by rapid test kit (RTK), hemagglutination inhibition test (HIT) and RT-PCR. Of the total 150 samples, 96 (64%) were positive and the remaining 54 (36%) were negative for NDV by RTK. Out of the 96 NDV positive samples by RTK, 77 (80.20%) were from chickens, 12 (12.5%) from pigeon and 7 (7.29%) from quail. In hemagglutination inhibition test (HIT), hyperimmune serum against NDV was used to confirm Newcastle disease virus that also showed 96 (64%) samples positive for NDV. In the RT-PCR, 100 (66.66%) samples showed positivity for NDV out of 150 samples. Total of 100 NDV positive samples by RT-PCR, 80 (80%) from chickens, 12 (12%) from pigeon and 8 (8%) from quail. Results of pathogenecity indices indicated that most isolates of NDV from chicken and pigeons were velogenic (MDT=48 hrs, ICPI=2.00 and IVPI=2.10) and all the isolate from quails were mesogenic (MDT=62 hrs, ICPI=1.20 and IVPI=1.10) in nature. Findings of the study closely indicated that velogenic strain of NDV was predominant in chickens compared to that of other two species. Present study also indicated that both velogenic and mesogenic strains of NDV are circulating among the three species of farm birds in Bangladesh.

KEYWORDS : NDV, RTK, HIT, RT-PCR

Newcastle disease (ND) is highly contagious viral disease of many domestic, semi-domestic and wild species of birds throughout the world which is caused by an enveloped, single stranded, negative sense and non-segmented genomic Ribonucleic Acid (ssRNA) virus. Newcastle disease virus (NDV) belonging to the genus *Rubulavirus* of subfamily *Paramyxovirinae* and family *Paramyxoviridiae* (Barbezange and Jestin, 2005). The genome of NDV consists of 15,186 bases which encodes six structural proteins in a single open reading frame (ORF) and arranged in the order from 3' to 5' as -N-P/C/V-M-F-HN-L- (Gohm et al., 2000). Based on the pathogenic and virulence properties, strains of NDV are distinguished into five pathotypes: viscerotropic velogenic viruses (vvND), neurotropic velogenic viruses (nvND), mesogenic viruses, lentogenic viruses, and asymptomatic enteric viruses causing gut infection but no apparent disease. In the context of Bangladesh, ND of poultry is caused mostly by velogenic strains of NDV rather than mesogenic or lentogenic (Islam et al., 1994). According to Chowdhury et al. (1982), Newcastle disease is responsible for at least 40-60% of total of poultry mortality in Bangladesh and is responsible for high economic losses every year. The rate of mortality in young and adult chickens due to very virulent (vvNDV) varies from 80-100% respectively. Of the farm birds chickens are highly susceptible, while ducks and geese can be infected and show few or no clinical signs with the same strain. The clinical signs of ND include depression, diarrhea, prostration, edema of the head and wattles. In the vvND, clinical signs often begin with restlessness, increased respiration ending with death. In some circumstances, with extremely virulent viruses the disease may result in sudden death. Therefore, present study was designed to confirm circulative pathotypes of NDV in the farm birds which will help to take necessary steps to develop preventive measure against the deadly disease of farm birds in Bangladesh.

MATERIALS AND METHODS

Cloacal swab samples were collected from dead farm birds of Bangladesh namely commercial broiler, layer, native birds, pigeon and quail. The samples were collected and transported at 4°C in the ice box maintaining cool chain. For initial screening, the tests were performed with the help of RTK (Anigen Rapid NDV 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 band for the NDV Ag. The presence of two color bands on

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the NDV Ag test area indicated positive result of Newcastle disease virus. Then the hemagglutination Inhibition (HI) test was performed for all samples using hyperimmune sera against NDV. Finally, all the samples those were found positive and negative for NDV in RTK and HI tests were further confirmed by reverse transcription polymerase chain reaction (RT-PCR) using gene specific primers against ND viruses after extraction of viral mRNA from cloacal swab samples. Reference virus velogenic (vvNDV/BD/2000) and mesogenic (Mukteswar strain) were used from the repository of the Department of Microbiology and Hygiene.

Haemagglutination (HA) test

Micro haemagglutination test was performed in a V-bottom 96 micro-well plate to determine HA units (4HA/50l). 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 0.5 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 all the V-bottom well and in HA positive case, clumping of red blood cells at the bottom of the V-bottom wells were showed in micro wells 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 of the test.

Haemagglutination Inhibition (HI) test

The HI test was performed to determine the inhibitory activity of hyperimmune sera against NDV for each of the HA positive cloacal swab samples collected from the chickens (broiler, layer and native), pigeon and quail. The test was conducted by using constant 4 HA unit antigen and increasing serum dilution method (beta procedure) following the procedure described by Reed and Muench (1938).

Molecular Detection

Primer Used

A set of oligonucleotide primers, forward (NDVF) - 5́-GCAGCTGCAGGGATTGTGGT-3́ (nucleotide position 158-177) and reverse (NDVR) - 5-TCTTTGAGCAGGAGGATGTTG-3́ (nucleotide position 493-513) as designed and used by Nanthakumar et al. (2000) were used for the amplification of 356 bp amplicons corresponding to the cleavage activation site of F gene of NDV.

RNA Extraction

RNA extraction was done in the class-100 laminar airflow in the laboratory of the Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh. The genomic viral RNA of NDV was extracted from 140.0 µl of cloacal swab samples of all the samples using the QIAamp mini RNA extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. A volume of 4 µl of eluted RNA of NDV and 8.3 µl DEPC were taken into individual PCR tube and mixed properly with the help of the minispin. The tubes were then placed in a forty eight wells thermocycler (Mastercyclear, 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 the vRNA. Meanwhile, for the synthesis of cDNA from RNA of NDV, the 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 NDV placed into the thermocycler and followed the thermal profile as 42°C for 60 minutes followed by 85°C for 5 minutes for cDNA synthesis.

For the synthesis of DNA from cDNA of NDV, reaction mixture was 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 of NDVF (100 pmol) 0.8 µl, NDVR (100 pmol) 0.8 µl, cDNA 1.5 µl. and DEPC 37.7µl

for NDV 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 extension at 60°C for 10 minutes. PCR product was allowed to run through by 2% Agarose NA gel electrophoresis and visualized by UV transilluminator.

Determination of Pathogenecity Indices of the Isolate

The mean death time with the minimum lethal dose (MDT/MLD) was determined with 9- 10 days-old chicken embryos as described by Hanson and Brandly (1955). The intracerabral pathogenecity index (ICPI) in day-old chicks and intravenous pathogenecity index (IVPI) in six-week- old chickens were determined according to Allan et al. (1978b).

RESULTS AND DISCUSSION

RTK

A total of 150 cloacal swab samples were subjected for RTK for the presence of NDV. Out of 150 samples, 96 (64%) were positive for NDV and the remaining 54 (36%) were negative by RTK. A 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 NDV. Among the 90 samples, 77 (85.55%) samples were positive for NDV and remaining 13 (14.44%) samples were negative for NDV.

A total of 30 cloacal swab samples were collected from pigeon and all the samples were subjected for RTK for the presence of NDV. Among the 30 samples, 12 (40%) were positive for NDV and the remaining 18 (60%) samples were negative for NDV. A total of 30 cloacal swab samples were also collected from quail and all the samples were subjected for RTK for the presence of NDV. Among the 30 samples, only 7 (23.33%) samples were positive for NDV and remaining 23 (76.66%) samples were negative for NDV.

HI test

All of the 150 cloacal swabs samples suspension those were positive and negative for NDV by RTK were subjected to HI test using hyperimmune sera against NDV. Before performing HI test, HA titration of each of the field sample was done to determine 4 HA unit. In HI test, 96 (64%) samples were positive for NDV those were completely inhibited by hyperimmune sera against NDV and remaining 54 (54%) samples were negative for NDV.

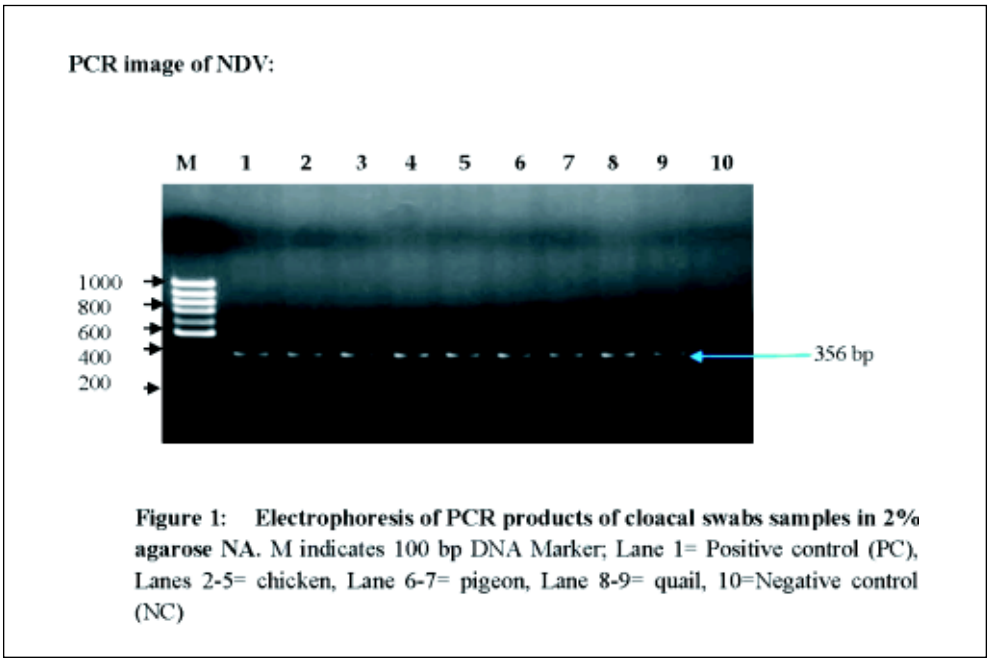

Figure 1: Electrophoresis of PCR products of cloacal swabs samples in 2% agarose NA. M indicates 100 bp DNA Marker; Lane 1= Positive control (PC), Lanes 2-5= chicken, Lane 6-7= pigeon, Lane 8-9= quail, 10=Negative control (NC)

Table 1 : Results of Pathogenicity Indices

Species of birds	Total Samples (150)	RT-PCR Positive samples (100)	Pathogenecity indices			
			MDT(hrs)	ICPI	IVPI	Pathotype
Broiler	30	29	52	2.00	2.10	Velogenic
Chicken Layer	30	24	50	2.00	2.10	
Native	30	27	48	2.00	2.10	
Pigeon	30	12	50	2.00	2.10	Velogenic
Quail	30	8	68	1.10	1.10	Mesogenic
Reference strain (vv NDV/BD)			48	2.00	2.10	Velogenic
Reference strain (Mukteswar)			62	1.20	1.10	Mesogenic

RT-PCR

All the 150 swab samples were further subjected to molecular detection method for confirmation by RT-PCR using gene specific primer set against NDV. The result of RT-PCR indicated that 100 (66.66%) samples were found positive for NDV out of 150 samples. RT-PCR product of NDV was found to show specific bands on 356 bp on 2% Agarose NA gel electrophoresis (Figure 1).

Pathogenecity Indices

The pathogenicity indices of the NDV isolates were determined by using 9-10 days old embryonated chicken eggs, day-old-chicks and 6-weeks-old chicken. In the MDT test, it was noticed that the isolate killed all the embryos within 52 hours of post infection. For ICPI test the isolate was found to kill all the embryos within 68 hours of post infection. In IVPI test the velogenic strain of NDV kill all the 6-weeks-old chicken within 72 hours and the mesogenic strain killed more than 60% within 6 days post infection when inoculated intravenously (Table 1). Based on the pathogenecity of the present field isolates that collected from chicken and pigeon were considered as velogenic and from quail were considered as mesogenic.

DISCUSSION

Present study was undertaken to detect the pathotypes of Newcastle disease viruses from three different species of dead farm birds (chicken, pigeon and quail) and also for sero-type determination of circulating NDV prevalent in Bangladesh.

In this study, a total of 150 cloacal swab samples were collected from three different species of farm birds during the period January to September 2014 for sero-type determination of NDV through initial screening by RTK, followed by serological (HI) test and molecular detection method.

Initial screening of all the 150 samples was performed for the presence of NDV in the cloacal swabs of farms birds by using the RTK. Result of RTK revealed that out of 150 cloacal swab samples of three different species of farm birds, 96 (64%) were positive for NDV and the remaining 54 (36%) were negative by RTK in this study.

A total of 96 positive samples by RTK, 77 (80.20%) were positive from chickens (27 from broiler, 24 from layer and 26 from native chicken), 12 (12.5%) from pigeon and 7 (7.79%) from quail. Overall results of RTK also indicates that the prevalence of NDV was only confined among the three species of farm birds namely chicken, pigeon and quail in this study. The results of RTK of the present study similarly agree with the findings of Malek (2009). Failure of RTK for the detection of NDV from the rest 54 field samples is not very clear but the death of the birds may be due to other reasons.

All of the 150 swab samples those were subjected for initial screening by RTK were further confirmed by HI test using hyperimmune sera against NDV present in the field samples in this study. The results of HI test indicated that 96 (64%) samples were also positive for NDV. Of the 96 (64%) NDV positive samples, 77 (80.20%) were positive from chickens (27 from broiler, 24 from layer and 26 from native chicken), 12 (12.5%) from pigeon and 7 (7.29%) from quail. Results of this study highly agree with the findings of Seal et al. (2000) who successfully using polyclonal serum raised against NDV by HI test to detect NDV directly from cloacal swab samples using field

samples from different species of farm birds.

All of the 150 swab samples which were positive and negative for NDV were further subjected to genome detection by RT-PCR using gene specific primers of NDV. In the molecular detection method (RT-PCR), 100 (66.66%) samples showed positivity for NDV. A total of 100 (66.66%) NDV positive samples by RT-PCR, 80 (80%) were positive from chickens (29 from broiler, 24 from layer and 27 from native chicken), 12 (12%) from pigeon and 8 (8%) from quail. The results of RT-PCR of the present study highly agree with the findings of Nanthakumar et al. (2000) and Creelan et al. (2002). In their study, they also stated the same results by using gene specific primers of NDV for the detection of the viruses from cloacal swab samples from different species of farm birds.

Results of the pathogenicity indices of the viruses of the present study revealed that most isolates of the recent field viruses were highly virulent and few isolates were moderately virulent strains of NDV. Based on the pathogenicity of the present field samples that collected from chicken and pigeon were considered as velogenic and the isolates from quail were considered as mesogenic. The properties which were considered to be velogenic and mesogenic of the field sample correlated with the criteria mentioned by several investigators (Banergee et al., 1994; Creelan et al., 2002).

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