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Molecular Detection, Biological Characterization and Evaluation of Protective Potentiality of a Velogenic Strain of Newcastle Disease Virus Isolate of Bangladesh

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Authors' contributions

This study was carried out in collaboration with all authors. Author MGH searched the literature, designed and performed the experiments and wrote the first draft of the manuscript. Author SS supervised the entire study. Authors SS, MAI and MMA helped to design and conduct the experiments and in preparing the manuscript. Author SA helped in data analyzing and manuscript preparation. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: The aim of this study was to isolate and characterize Newcastle disease virus (NDV) from recent outbreaks in Bangladesh and protective potentiality evaluation of a velogenic NDV strain.
Methodology: A total of 19 lung tissue samples were collected from dead layer chickens of clinically suspected Newcastle disease (ND) cases. Ten days old embryonated chicken eggs, day-

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old chick and six-week-old sero-negative chickens were used for the isolation and pathotype determination of the virus. Hemagglutination (HA), hemagglutination inhibition (HI) tests using anti-APMV-1 polyclonal serum were used for primary identification and reverse transcription polymerase chain reaction (RT-PCR) was carried out for the confirmation of the isolated viruses by amplification of F gene of NDV using gene specific primers. Three, out of eleven isolates of NDV were subjected to pathotype determination by mean death time (MDT), intracerebral pathogenecity index (ICPI) and intravenous pathogenecity index (IVPI). One of the isolates of NDV of the year 2012 was selected as vaccine candidate to determine its immunogenicity. ND vaccine was prepared by inactivating virulent Newcastle disease virus (vNDV) with 0.1% formaldehyde and adjuvanted with 40% aluminium hydroxide gel.

Results: Of the 19 samples total eleven isolates were initially identified as NDV by HA and HI tests and finally confirmed by RT-PCR. Results of MDT, ICPI and IVPI indices indicated that all the isolates of NDV of 2011 and 2012 were velogenic in nature. The inactivated vaccine produced satisfactory level of antibody titre at 72 days of post vaccination and revealed 100% protection during challenge experiment with 2 egg lethal dosage, ELD_{50}/bird against both the velogenic strains of NDV.

Conclusion: The frequent outbreaks of ND caused by vNDV in Bangladesh could easily be controlled by using inactivated vaccine prepared with velogenic strain of NDV.

Keywords: NDV; RT-PCR; pathotype determination; vaccine; hemagglutination inhibition.

1. INTRODUCTION

Newcastle disease (ND) is a highly contagious and widespread viral disease which causes severe economic losses in domestic poultry, especially in chickens [1,2]. The Newcastle disease is caused by avian paramyxovirus serotype 1 (APMV-1) belonging to the genus Avulavirus in the family of Paramyxoviridae [3]. Newcastle disease virus is a single-stranded, negative-sense enveloped RNA virus and the viral genome is 15,186 bps long and contains six genes encoding major polypeptides: nucleocapsid protein (NP), phosphoprotein (P), matrix-protein (M), fusion (F) protein, hemagglutinin- neuraminidase (HN) and large RNA-dependent polymerase protein (L) [4,5].

For confirmation of ND, the OIE prescribes isolation of NDV in embryonated chicken eggs and their identification using haemagglutination (HA) and hemagglutination inhibition (HI) tests with a NDV-monospecific antiserum [6]. Reverse transcription-polymerase chain reaction (RT-PCR) has also been established to identify NDV of known and unknown samples [7-9]. Strains of NDV are categorized into three pathotypes based on their pathogenicity in chickens, viz: (i) highly virulent (velogenic), (ii) intermediate virulent (mesogenic), and (iii) nonvirulent (lentogenic) [1]. The pathogenicity of any isolate of NDV can be assessed by the determination of mean-death-time (MDT) in chicken embryos, the intracerebral pathogenicity index (ICPI) in day-old chicks and the intravenous pathogenicity index (IVPI) in 6-week-old chickens [6].

Vaccination program is being used widely for the prevention and control of ND in Bangladesh, but the disease is still enzootic throughout the country and remains a constant threat to the commercial poultry. Sometimes, reports of severe outbreaks of ND are made even after regular vaccination of chicken with live NDV vaccines prepared from mesogenic and lentogenic strains [10]. There might be significant difference in the prevailing strain of NDV and vaccine viruses in their biology, serology and genetics, which are considered as the main reasons for the frequent outbreaks of this disease in the vaccinated poultry flocks currently in Bangladesh [11]. Reports on molecular and pathological characteristics of NDVs circulating in Bangladesh are very poor.

This study has been designed for molecular detection and biological characterization of NDV isolated from dead layer chickens of recent outbreaks in Bangladesh and determination of immune response of the inactivated NDV vaccine prepared from the velogenic strain of recent isolate of NDV.

2. MATERIALS AND METHODS

2.1 Sample

A total of 19 lung tissue samples were collected from dead layer chickens of clinically suspected ND of three outbreaks areas, Shesmore (24.718006, 90.443983), Boyra (24.71125868, 90.43458225) and Sutiakhali (24.697106,

90.45308948) of Mymensingh District of Bangladesh during 2011-2012 (Table 1). The samples were preserved at -80℃ until use.

2.2 Reference NDV

Velogenic strain of NDV (NDV/DBD/1/2008) used as positive control for RT-PCR and for challenge infection as well. The virus obtained from the repository of the virology laboratory, Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh-2202.

2.3 Chicken Eggs

9-10 days-old fertile eggs from sero-negative breeder flock of layer chickens were purchased from Phenix Hatchery Ltd., Gazipur, Bangladesh for the isolation of viruses and MDT study.

2.4 Experimental Chicks

A total of twenty four (24) day old (Layer BB300) chicks were obtained from the Phenix Hatchery Ltd., Gazipur, Bangladesh. The chicks were reared with adlibitum supply of feed and water for 12 weeks maintaining under strict biosecurity in a well-ventilated experimental poultry shed of the Department of Microbiology and Hygiene, BAU, Mymensingh according to university animal care guidelines.

2.5 Virus Isolation and Identification

Inoculum preparation, virus isolation and identification were carried out as per the standard methods described in OIE [6].

2.6 Viral RNA Extraction and F Gene Amplification

The genomic viral RNA was extracted from the HA and HI positive AF by using QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The RNA was eluted using 60 µl of elution buffer and stored at -80℃ until used. The extracted NDV genomic RNA was used to generate cDNA by RT-PCR. The oligonucleotide primers forward (NDV/Fa:5'-GTCAACATATACACCTCATCCCAGACAGG-3') and reverse (NDV/Ra:5'-CTGCCACTGCTAGTTGGGATAATCC-3') were used to amplify the F gene of NDV. The expected size of the RT-PCR amplified DNA fragment is 387 bp in length. All the RT-PCR products were run through 2% agarose gel electrophoresis and stained with 0.01% ethidium bromide and compared with molecular marker (100 bp DNA marker) by visualizing with ultraviolet (UV) trans-illuminator.

2.7 Pathogenicity Indices of the Field Isolates of NDV

Three isolates (Table 2) of NDV were subjected to pathotype determination by MDT, ICPI and IVPI. The mean death time or the minimum lethal dose (MDT/MLD) was determined using 9-day-old chicken embryos following the methods described by Hanson and Brandly [12]. The intracerebral pathogenicity index (ICPI) was done in day-old chicks following the method described in OIE [6]. The intravenous pathogenicity index (IVPI) was determined in 6-week-old chickens following the method described previously [13].

2.8 Experimental Preparation of an Inactivated Vaccine with Velogenic Strain of NDV

Of the three characterized velogenic strains one NDV (NDV/MBD/8/2012) was selected as vaccine candidate to determine its immunogenicity against vNDV. The infectivity titre (ELD_{50}) of NDV was determined by inoculating serial 10-fold diluted (10^{-1} to 10^{-10}) NDV positive AF, into embryonated chicken egg (ECE) and the virus titre was calculated following the method described by Reed and Muench [14]. Infective AF containing field isolate of NDV having ELD_{50} $10^{8.7}$/ ml of AF was inactivated by 0.1% formalin at a final concentration of 1:1000 following the method described by Koppad *et al.* (2011). Inactivation of NDV was checked by two serial passages in ECE resulting in live embryos with no HA activity in their AF. A 40% aluminium hydroxide gel was added with the inactivated NDV to prepare NDV vaccine following the method described by Tizard [15].

2.9 Sterility and Safety Tests of the Experimentally Prepared Inactivated NDV Vaccine

The sterility and safety tests of the inactivated NDV vaccine was done according to the OIE by using blood agar media and also in six 5-day-old chicks respectively [6].

2.10 Experimental Immunization, Serology and Challenge Studies

The experimental chicks were divided into two (2) major groups (A and B) each consisting of 12 chicks. Chicks of group A immunized thrice with inactivated NDV vaccine through intramuscular (i/m) route at 5, 28 and 56-days of age at the doses rate of 0.25 ml, 0.5 ml and 1 ml/ bird respectively. Chicks of group B were kept as unvaccinated control. Efficacy of the inactivated NDV vaccine was then evaluated by measuring HI antibody titre of sera samples collected from chicken at day 4, 27, 55 and 72 respectively as pre and post vaccinations following the method described by Anon [16].

The chickens of vaccinated group (Group A) were again divided into two sub-groups namely A_1 and A_2 and the unvaccinated group (Group B) into two sub-groups namely B_1 and B_2. Chickens of sub-groups A_1 (Vaccinated) and B_1 (Unvaccinated) were challenged with reference NDV (NDV/DBD/1/2008) and chickens of sub-groups A_2 (Vaccinated) and B_2 (Unvaccinated) with recent isolate of NDV (NDV/MBD/8/2012) through intranasal (i/n) route at a dose rate of 0.1ml ($2ELD_{50}$) per bird which correspond about 100% mortality in chickens of 10 weeks of age [17].

2.11 Analysis of Data

Data (HI titre) were expressed as Mean ± SE and the differences of serum antibody titres among the groups of chickens were compared using one-way ANOVA. Statistical analysis was performed using SPSS software version 17 and the significant level was set as $P \leq 0.05$. Survival rate of chickens after challenge experiment were analyzed by Mantel-Cox log rank test.

3. RESULTS

3.1 Virus Isolation and Identification

A total of eleven NDV isolates were isolated from 19 field samples. Firstly all HA positive AF was harvested and preliminary confirmed by HI test with anti-APMV-1 polyclonal serum and then RT-PCR using F gene specific primers of NDV for final confirmation of the virus (Fig. 1). The new isolates of NDVs were designated as NDV/MBD/1-11/2011/2012 (Table 1).

Table 1 showing isolation and identification of NDV from 19 lungs tissue samples collected from three different areas of Mymensingh district in Bangladesh.

3.2 Pathogenicity indices

Results of the mean-death-time (MDT), intracerebral pathogenicy index (ICPI) and intravenous pathogenicity index (IVPI) of the three representative field isolates (NDV/MBD/1/2011, NDV/MBD/5/2012 and NDV/MBD/8/2012) of NDVs indicated that all the isolates were velogenic in nature (Table 2).

3.3 Sterility and Safety Tests of the Inactivated NDV Vaccine

The inactivated NDV vaccine was sterile and safe after inoculation into blood agar media and administration of double dose of the vaccine in commercial layer chicks (either intramuscular or intravenous) respectively.

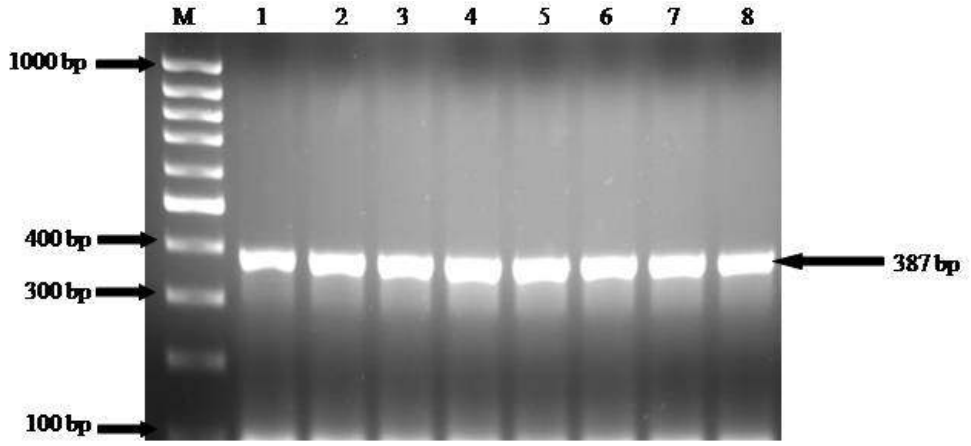

Fig. 1. Amplification of F gene fragment (387 bp) of NDV by RT-PCR. The lanes in the photograph showing, M=Marker (100 bp), Lane-1= Positive Control and Lane 2-8= NDV isolates

Table 1. Isolation and identification of NDV from field samples

Sampling sites	Number of samples	Isolation in avian embryos	HA positivity	HI positivity	RT-PCR positive	Total isolates	Nomenclature of the virus isolate
Shesmore Mymensingh	6	4	4	4	4	4	NDV/MBD/1-4/2011
Boyra of Mymensingh	6	3	3	3	3	3	NDV/MBD/5-7/2012
Sutiakhali of Mymensingh	7	4	4	4	4	4	NDV/MBD/8-11/2012
Total isolates						11	11

Table 2. Pathotype determination of field isolate of NDV. The MDT, ICPI and IVPI values of the three representative isolates, viz NDV/MBD/1/2011, NDV/MBD/5/2012 and NDV/MBD/8/2012 of NDV

Field isolates	MDT	ICPI	IVPI	Pathotype
NDV/MBD/1/2011	55.2 hrs	1.80	2.70	Velogenic
NDV/MBD/5/2012	58.8 hrs	1.83	2.67	Velogenic
NDV/MBD/8/2012*	57.6 hrs	1.81	2.69	Velogenic

** This isolate of NDV used for immunization and challenge experiment in this study*

3.4 HI Antibody Titre of Serum Samples of the Vaccinated and Unvaccinated Control Chickens

The serum antibody titre of vaccinated chickens was gradually increased until 72 days of age following primary, secondary and tertiary vaccinations whereas maternal antibody titres of unvaccinated chickens was decreased dramatically at 72 days of age (Fig. 2).

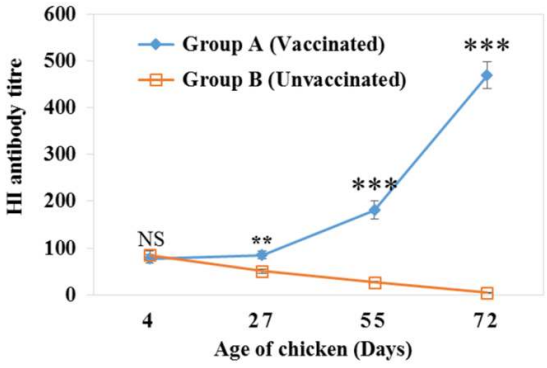

Fig. 2. HI antibody titer of chickens of vaccinated and unvaccinated control groups

The chickens of group A were vaccinated with inactivated NDV according to materials and methods whereas chickens of group B were kept as unvaccinated control. Sera samples were collected at the indicated time points and subjected for antibody titre analysis by HI. The data are presented as average (n=12 chickens/group) of serum HI antibody titre of the both vaccinated and unvaccinated chickens with error bar presenting SEs. HI = Haemagglutination inhibition, SE = Standard error, NS = Non significant, ** =*P* <0.01 and *** =*P* <0.001 by one-way ANOVA.

3.5 Protection Test

All the vaccinated chickens of the sub-groups A_1 and A_2 exhibited no signs of illness and survived following challenge infection with both the reference and field isolates of velogenic strain of NDV (NDV/MBD/8/2012) throughout the 10 days period of observation. Besides this, the control chickens of the sub-groups B_1 and B_2 challenged with both the reference and field isolates of velogenic strain of NDV (NDV/MBD/8/2012) at the same day started to die from day 3 and all birds died within day 6^{th} of challenge experiment. Therefore, the results suggested that vaccine prepared from velogenic NDV strain conferred 100% protection following virulent challenge (Fig. 3).

The line with rectangular marks (Blue color) and the line with square marks (Magenta color) representing the survivability rate of immunized groups (sub-groups A_1 and A_2). The line with triangle marks (Green color) and the line with cross marks (Violet color) representing the

death rate of non-vaccinated groups (sub-groups B_1 and B_2). (n=06 chickens/sub-group). ***=*P* <0.001 by Mantel-Cox log rank test.

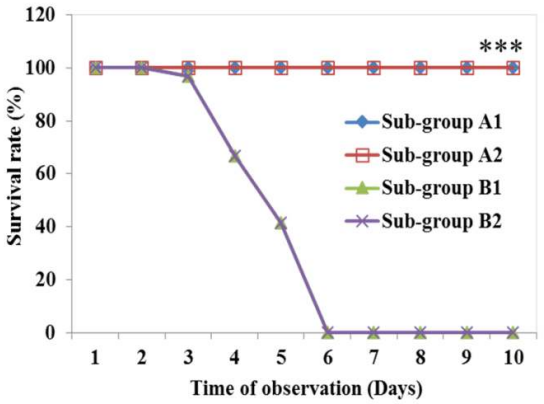

Fig. 3. Survival rate of chickens of vaccinated and unvaccinated control groups

4. DISCUSSION

A total of 19 lungs tissue samples were collected from dead layer chickens of clinically suspected ND of three outbreaks areas of Mymensingh district of Bangladesh. Out of 19 samples, 11 were found positive for NDV through isolation using 10 days old embryonated eggs of sero-negative hens.

Firstly, the HA positive AF was harvested and subjected to HI test. All the 11 HA positive AF were found to be positive in HI test and these findings have close similarity with other investigators where they isolated NDV from field samples and indentified by HI test [18,19]. Eleven isolates of NDV, preliminary confirmed by HI test were subjected to RT-PCR for further confirmation by using F gene specific primer of NDV (Fig. 1). The expected sized band of RT-PCR-amplified DNA fragment was found at around 387 bp. This molecular detection of NDV by using F gene specific primer has stronger similarity with the findings of others [8,9].

Three (NDV/MBD/1/2011, NDV/MBD/5/2012 and NDV/MBD/8/2012) of the eleven isolates of NDV were subjected to pathotype determination by MDT, ICPI and IVPI**.** The results of MDT, ICPI and IVPI of the three representative field isolates of NDV indicated that all the recent isolates of NDV belong to the velogenic group which also correlates with the findings of several investigatiors [20,21].

One representative velogenic strain (NDV/MBD/8/2012) of the three field isolates of NDV was used as a vaccine candidate to check its immunogenicity and protective potentiality of chickens against the circulatory velogenic strain of NDV. The mean ±SE of HI antibody titres of chickens (Fig. 2) vaccinated thrice with inactivated NDV vaccine through i/m route at 5-, 28- and 56-days of age were found to be increased from day 4 to day 72 of age and the HI titre was increased significantly at 55- and 72-days (*P* <0.001, day 27 vs. day 55 and day 55 vs. day 72) of age and such results have close relationship with the previous findings [22-24]. The serum antibody level of chickens following primary vaccination did not show any significant production of HI antibody. This lower level of antibody production was due to presence of high level of maternally derived antibody (MDA) as the chicks used in this study had comparatively high MDA and this finding correlates with the findings of other scientists [17,25].

The antibody titre analysis of sera samples obtained from the unvaccinated chickens at 4-, 27-, 55- and 72-days of age showed that the antibody titres were gradually decreased from day 4 to day 76 of age (Fig. 2). This finding is in agreement with the findings of Sarkar *et al* where the author found that MDA may persist up to 76 days of age in the layer chickens [17].

The vaccinated and unvaccinated chickens were challenged at 72 days of age (16 days after tertiary vaccination) with both the velogenic strains of the field (NDV/MBD/8/2012) and reference NDVs. The chickens vaccinated thrice with the inactivated NDV vaccine conferred 100% protection and this results are in agreement with the findings of Saha *et al.* and Zhao et al. [10,26]. On the other hand, chickens of unvaccinated control group failed to protect any chickens following challenge infection (Fig. 3).

The lower survival rate (50-60%) of commercial chickens vaccinated with live NDV vaccines may be due to variation in antigenicity between the field and vaccines viruses. Another possibility of regular vaccination failure may be due to the improper maintenance of cool chain during transportation and storage of the live NDV vaccines. The highest rate of efficacy of the inactivated NDV vaccine prepared with local isolate may be due to the strong antigenic relationship between the field and vaccines viruses. Inactivated and adjuvanted NDV vaccine

revealed higher rate of protective potentiality due to slow release of the antigen.

5. CONCLUSION

The velogenic strain of NDVs are circulating among the poultry population of Bangladesh. The inactivated vaccine prepared with velogenic field isolate of NDV (NDV/MBD/8/2012) might induce satisfactory level of serum antibody after vaccination and provide 100% protection in chickens against the existing velogenic strain of NDV circulating in the Bangladesh.

ETHICAL APPROVAL

All authors hereby declare that they followed the Principles of laboratory animal care (NIH publication No. 85-23, revised 1985) as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate animal research committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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