Development of BHK-21 Cell line Adapted Inactivated Newcastle Disease Vaccine.

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ABSTRACT

The present study was undertaken to isolate and characterize Newcastle disease virus (NDV) from the outbreak of layer farms for the development of BHK-21 cell adapted inactivated vaccine. A total of 6 dead birds were brought to the laboratory from the outbreak area from which 18 samples (trachea, liver and brain from each) were collected. Among them 3 samples were found positive for NDV through chicken embryo inoculation followed by HA test. The MDT, ICPI, IVPI of all isolates was 54.4, 1.56, and 2.20 respectively which revealed that the field isolates were velogenic. The isolated viruses were confirmed as NDV by reverse transcription-polymerase chain reaction (RT-PCR) using fusion gene-specific primers. The isolated virus was used to infect the BHK-21 cell line. Later the BHK-21 cell adapted viruses were used to develop oil adjuvanted inactivated vaccine and were inoculated into chickens according to vaccination schedule. The MDA was very high (112±29.62) during BCRDV vaccination, which declined quickly (88±33.12). Before vaccination with experimental vaccine, the level of antibody titre (HI) was very low (9±4.65). After vaccination at 65th day through IM, $ELD_{50}=10^{9.7}$/ml with experimental vaccine, the highest HI titre in RDV vaccinated group was 160±59.25 whereas, experimental vaccinated group was 128±59.25, and control group was 7±4.65. ELISA antibody titres of all the groups were 2549.71 (RDV, LRI@0.5 ml/bird), 2450.37 (experimental@ 0.25ml/ bird), 2218.579 (experimental@ 0.50ml/bird), 2152.352 (experimental@1ml/bird) 1125.846 (control) respectively**.** The present study indicated that, BHK-21 cell adapted ND inactivated vaccine produced a satisfactory antibody titre along with conventional live RDV vaccine.

Key Words: NDV, RT-PCR, BHK-21 cell line, Inactivated Vaccine.

Received: 26th January, 2014. Accepted: 7th July, 2014

Introduction

Newcastle disease (ND) is a deadly viral disease of poultry all over the world since the time of first isolation in England in 1926. It is an acute, contagious infection of pet, free living and domestic birds. This disease is caused by a single stranded, enveloped, negative sense RNA virus belonging to the genus Avula virus of sub family Paramyxovirinae and family Paramyxoviridae (Barbezange and Jestin, 2005; Mayo, 2002). A variety of NDV isolates and strains have been recorded around the world (Alexander, 1991; Ballagi-Pordany et al., 1996; Rahman et al., 2004, Molia et al., 2011). Depending on the basis of pathogenicity, the NDV has been distinguished into five pathotypes such as viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic, and asymptomatic viruses (Alexander, 1997). In Bangladesh, frequent outbreak of ND is mostly due to velogenic strain of NDV (Chowdhury et al. 1982).

Effective control of ND relies on the use of safe and effective vaccines. Two live vaccines; BCRDV (F-strain for baby chick) and RDV (M-strain for adult) produced by the Directorate of Livestock Services (DLS), Dhaka are now being used countrywide regularly to control the disease in Bangladesh. Sometimes reports of severe outbreak of ND are noticed even after vaccination of chicken with the two live vaccines. Such vaccines and the program of vaccination have been found inadequate to protect chicken against ND in Bangladesh (Chowdhury et al. 1982). Other groups of researchers have conducted research on the ND vaccines and their efficacy (Kafi et al., 2003; Rahman et al., 2004; Shil et al., 2006; Samad et al., 2007; Khalil et al., 2011). Despite regular vaccination, the disease is found to appear every year in many poultry farm as epidemic form causing 40-60 percent of the total mortality of chicken in Bangladesh. Such a nature of the disease demands an effective vaccine to control the morbidity and mortality rate of chicken in Bangladesh. Therefore, the present study was undertaken for the production and to compare the level of antibody titres of experimental BHK-21 cell adapted inactivated ND vaccine with the conventional live virus vaccines (RDV, LRI).

Materials and Methods

### Isolation and propagation of viruses in avian embryos

A total of 6 dead birds died from suspected cases of NDA were brought to the laboratory from Mymensingh and the surrounding areas from which a total of 18 samples (trachea, liver and brain from each dead bird) were collected aseptically and inoculum were prepared (El-Yuguda et al., 2014; Murmu et al., 2014). Initially virus was propagated in chicken embryo according to the procedure described by Anon (1971). Briefly, the inoculum (0.2ml) of each sample was inoculated into 10-day-old embryonated chicken egg through allantoic cavity route. After inoculation the eggs were incubated at 37^0C with a humidified condition (50-60%) observed twice daily for mortality of the embryo. The embryos died due to ND virus were chilled followed by harvesting of the allantoic fluids and then preserved at -20^0C until further use. The presence of virus in allantoic fluid was confirmed by slide HA test using 2 % chicken RBC suspension. The lesions on the body of the embryos were examined and recorded.

Adaptation of virus in BHK-21 cell line

The cells those formed complete and confluent monolayer in the culture bottle within 24-72 hours were selected for infection with viruses. The BHK-21 cell line was infected with 1 ml of inoculum and was spread over the cell sheet by tilting for about 45-60 minutes for the establishment of better interaction. Then 5-10 ml of the maintenance medium (MEM supplemented with 2% fetal bovine calf serum) was added and followed by incubation of the bottle at 37^0C. The cells were examined twice daily under DIC microscope until cytopathic effect (CPE) was formed by inoculated virus.

Primers and RT-PCR for detection of NDV

In this study two primers forward: 5'-GCA GCT GCA GGG ATT GTG GT-3' nucleotide position (158-177) and reverse: 5'-TCT TTG GAG CAG GAG GAT GTT G-3' nucleotide position 493-513) described by Nanthakumar et al., (2000) were used. Total RNA from the allantoic fluid was extracted with Invisorb® Spin Virus RNA Mini kit as per
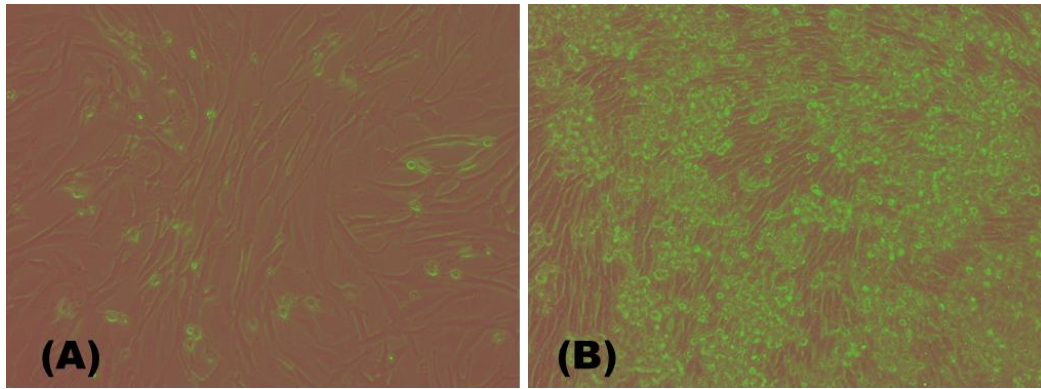
Figure 1. (A) Noninfected BHK-21 cell line, (B) NDV infected BHK-21 cell showing typical cytopathic effect (CPE): multinucleated giant cell

manufacturer's instructions. RT-PCR was carried out using Access RT-PCR system (Promega, USA). DNA amplifications were performed in a total volume of 50 μl containing 10μl 5X reaction buffer, 1μl dNTP mix (10mM), 10 pmol of each primer 1μl, 2μl 25mM MgSO4, 1μl AMV Reverse Transcriptase (5u/μl), 1μl *Tfl* DNA polymerase (5u/μl), 4μl RNA sample and nuclease-free water was added to a final volume of 50μl reaction mixture. For first Strand cDNA synthesis was conducted at 45°C for 45 minutes for reverse transcription (1 cycle), 94°C for 2 minutes for AMV RT inactivation and RNA/cDNA/primer denaturation (1cycle). The reaction mixture were thermocycled 40 times beginning with an initial denaturation step of 4min at 94 ºC. The temperature and time profile of each cycle was as follows: 94°C for 30 seconds (denaturation), 60°C for 1 minute (annealing), and 68°C for 2 minutes (extension). PCRs were finished with a final extension step of 68°C for 7 minutes and the products were stored at 4°. The PCR products were separated by electrophoresis in 1.5% agarose gel. The PCR products were visualized by UV transillumination after staining with 0.5 μg/ml ethidium bromide.

Preparation of inactivated vaccine

Infected BHK-21 cell culture fluid was taken in a sterile test tube. Appropriate amount of commercial formaldehyde (37%) was added to a volume of 25 ml of ND infected cell culture fluid and kept at room temperature for 24 hours to inactivate the virus particles properly. The inactivation was confirmed by inoculation of inactivated vaccine to BHK-21 cell line. After confirmation of inactivation of virus, the inactivated fluid was mixed with appropriate amount of liquid paraffin, Urlacil A, Tween 80 and mixed by magnetic stirrer which was used for immunization of bird. The dose used was 0.25 ml, 0.5 ml and 1.0 ml ($ELD_{50}=10^{9.7}$/ml) of inactivated vaccine/bird.

Vaccination trial

The vaccination trials were carried out in layer birds housed on experimental poultry shed in the department of Microbiology and Hygiene. A total of 150 birds were separated into 5 different groups A, B, C, D and E. Each group consists of 30 birds. The birds of A, B, C and D were vaccinated at 50th day with RDV (LRI) @ 0.5 ml/bird, experimental vaccine @ 0.25ml, 0.50 ml and 1ml per bird through intramuscular (IM) route, respectively. The birds of group E were unvaccinated control. The sera samples from all groups were collected at 7 days before vaccination and 15 days post vaccination including the control group. The collected sera samples were used to perform HI and ELISA tests to determine the antibody titer.

Determination of antibody titer by HI and ELISA tests

The sera samples were collected from all vaccinated and nonvaccinated birds and subjected to HI test to determine the antibody titer to the method of Anon (1971). To perform HI test the sera samples were heated into water bath maintaining 56ºC for 30 minutes. The AF was used as test virus after determination of. At first two fold dilutions of the sera were prepared using PBS which were mixed with 4 HA unit virus and the plate was left for one hour at room temperature followed by addition of 1% (v/v) cRBCs to each well for 45 minutes at room temperature. The result of hemagglutination inhibition was recorded by observing the formation of button at the bottom of the plates.

The antibody titer in the vaccinated and unvaccinated birds was determined by indirect ELISA test using commercially available NDV virus coated ELISA kit (as per manufacturer's protocol). The test was performed according to the instruction of the manufacturer company and the absorbance of the test samples and control in the micro-titer

plate was recorded at 405 nm using Multiscan Ex (Thermo, USA).

Interpretation of results

For the test result to be valid the mean negative control absorbance should be <0.30 and the difference between the mean negative control and the mean positive control should be >0.15. The NDV positive control has been carefully standardized to represent significant amounts of antibody to NDV in chicken serum. The relative amounts of antibodies in chicken serum can then be calculated by reference to the positive control. This relationship is expressed as Sample to Positive (S/P) Ratio.

Results

Clinical findings observed in NDV infected chickens

Suspected natural outbreak of ND in commercial layer farms from which the samples were originated accounted 100% morbidity with 85% mortality within 7 days of onset of clinical signs. The clinical signs were recorded including respiratory distress (gasping and coughing), nasal discharge, cloudiness of the cornea of the eye, greenish-white diarrheic feces, paralysis of legs and wings, tremors, severe depression and prostration followed by death. The post mortem lesions were also observed which were consisted of button like ulcer in the duodenum, petecheal haemorrhage in the colon, haemorrhage in the proventriculus and congestion and hemorrhage in the trachea and lungs.

Table 1: Post-vaccination HI titres of chicks of all groups at 65th day

Group of birds	Type of vaccine	Dose	Mean±SD
Prevaccination			8±5.32
Group-A	RDV	0.5ml ELD_{50} =$10^{9.7}$/ml	160±59.25
Group-B	ND killed	0.25ml ELD_{50} =$10^{9.7}$/ml	128±59.25
Group-C	Do	0.5ml ELD_{50} =$10^{9.7}$/ml	76±33.94
Group-D	Do	1ml ELD_{50} =$10^{9.7}$/ml	68±26.70
Group-E	Nonvacccinated		7±4.65

SD=standard deviation, RDV= Ranikhet disease vaccine, IM=Intra mascular, ND=Newcastle disease samples collected from each group of the chickens before and after vaccination respectively in this study. The pre-vaccination OD value was 0.54±0.17. After experimental vaccination the OD value of all groups were 0.94±0.13, 0.90±0.117, 0.82±0.15, 0.76±0.11, 0.45±0.19 respectively. From the OD value, S/P value of five groups were calculated as 0.77, 0.74, 0.67, 0.65, 0.34 and antibody titres were determined as 2549.71, 2450.37, 2218.579, 2152.352, 1125.846 respectively (Table 2).
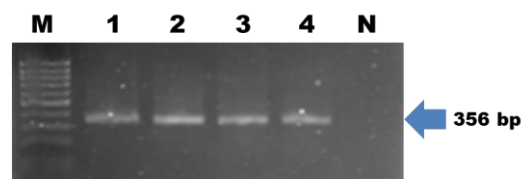
Fig. 2. Agarose gel electrophoresis of 356 bp PCR products of NDV field isolates and reference strains. Here lane M: 100 bp DNA Ladder, Lane 1: Reference (Vaccine strain: Mukteswar), Lane 2:Isolate1, Lane 3: Isolate 2, Lane 4: Isolate 3, Lane N: Negative control.

Table-2: Comparison of OD value of different groups of chicken (15 days of post vaccination)

Group of birds	Type of vaccine	Dose (ml)	Mean OD±SD	Mean S/P value ±SD	Mean antibody titre ±SD
Pre-vaccination	N/A	N/A	0.54±0.17	0.34	1125.846
Group-A	RDV	0.5, $ELD_{50}/ml=10^{9.7}$	0.94±0.13	0.77	2549.71
Group-B	ND killed	0.25, $ELD_{50}/ml=10^{9.7}$	0.90±0.12	0.74	2450.37
Group-C	ND killed	0.5, $ELD_{50}/ml=10^{9.7}$	0.82±0.15	0.67	2218.579
Group-D	ND killed	1.00, $ELD_{50}/ml=10^{9.7}$	0.76±0.11	0.65	2152.352
Group-E	Nonvaccinated	N/A	0.45±0.19	0.34	1125.846

RT- PCR detection of NDV

Newcastle disease virus was detected in 3 of the 18 field samples as well as in the vaccine strain by RT-PCR. Of the positive samples, all samples had a single band of 356 bp by RT-PCR. There was no amplification in the negative control with RT-PCR (Fig. 2).The total RNA was extracted from allantoic fluid using Invisorb® Spin Virus RNA Mini kit. It yielded sufficient pure RNA which could be used directly for reverse transcription polymerase chain reaction (RT-PCR). The methodology of RT-PCR as described by Nanthakumar *et al.* (2000) was found suitable for amplification of 356 bp of F region of NDV. The specificity of RT-PCR was confirmed by absence of amplification in unvaccinated apparently healthy tissue sample taken as negative control.
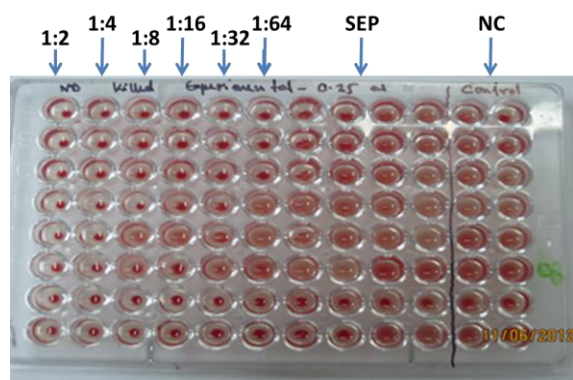

Fig 3. Determination of HI titre. SEP=Serum End Point, NC=Negative control

BHK-21 cell line adaptation of NDV

The embryos infected with fields isolates died within 72 hours of inoculation and allantoic fluid (AF) were collected from the dead embryos. In BHK-21 cell line the viruses produced characteristics cytopathic effects such as polykaryocytosis, syncitium formation, rounding of cell, and ghost cells at 24 hours and 48 hours (Fig. 1 A and B) hours of post infection. Initially the presence of virus in AF and tissue cultured fluids (TCF) were detected by direct HA test. All viruses of 3 samples were grown in chicken embryo and BHK-21 cell line exhibited HA activity. The isolated viruses were confirmed as NDV by RT-PCR of NDV specific fusion (F) gene. Upon the pathogenicity tests, the value of mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) value were found 54.4, 1.56, and 2.20 respectively which represent the isolates as velogenic.

Determination of antibody response by HI and ELISA tests

The pre-vaccination HI titers were determined at 65th day and no detectable Ab against NDV was observed in their sera. After experimental vaccination detectable HI Ab were found; whereas no detectable Ab was found in nonvaccinated flocks (Table 1& 2 and Fig. 3).

Discussion

Newcastle disease is a global disease of enormous economic importance. The virus is capable of infecting many avian species with a marked effect on the poultry industry, principally due to mortality but also due to the effects on the quality and quantity of meat and eggs produced by affected birds.

The clinical manifestations and gross lesions seen in the layer chickens of all selected farms suspected as viscerotropic velogenic NDV similar to those reported previously (Doyle, 1935). NDV were similar recorded

as listlessness, dyspnea, weakness, presence of greenish diarrhoea ending with prostration and death. The post-mortem lesions in various organs of chickens were button like ulcer in the duodenum, ulcer in the proventiculus, hemorrhage in the caecal tonsil, petechial hemorrhage in the colon, mottled red hemorrhage and congestion in the spleen and kidney and congestion and hemorrhage in the lungs which are typical lesions of ND as described by Ojok and Brown (1996).

The rate of isolation of NDV was 83% and 100% in avian embryos and cell line respectively. The isolation rate was lower in avian embryos might be due to presence of maternal antibody in embryonated eggs. The growth of virus in BHK-21 cell line were confirmed by the presence of cytopathic effects (CPE) which are the characteristics of syncitia formation, rounding of cell and multinucleated giant cells which are typical for NDV which are close agreement with the findings of Gerganov (1978) and Ravindra *et al.* ,(2009).

The pathogenicity of the isolated viruses was determined by MDT, ICPI and IVPI. The MDT of avian embryos was 54.4, which indicate that all the field isolates of NDV were velogenic. On the contrary, the value of ICPI in day-old chicks was 1.56 which clearly represents that the isolates were velogenic NDV. The velogenic nature of the recent field isolates of NDV was also confirmed by the result of determination of IVPI which was 2.20. All the above result is supported by Qin *et al.* (2006). From the result of these pathogenecity tests it could be concluded that the field isolates of the present study were highly pathogenic (velogenic).

Due to inadequate storage and transport facility in Bangladesh we have adopted to develop killed ND vaccine which was inoculated into layer birds to observe the level of Ab production. Following hemagglutination-inhibition (HI) test, 100% of the sampled birds were found to have protective levels of antibodies against NDV. This finding support some other reports which found protective antibodies attained in less than 100% of vaccinated birds. Foster et al. (1999) found that at least 70% of the chickens vaccinated using thermo-tolerant ND-I2 vaccine would be protected against challenge with virulent virus.

In the present study the antibody titre in the vaccinated and nonvaccinated birds were determined by HI and ELISA tests. The antibody titre in both prevaccinated and nonvaccinated birds were very low as compared to the vaccinated birds' *i.e.* The killed vaccine showed a satisfactory antibody titer (128±59.25) where the protective label is 128 or more along with conventional live RDV vaccine. These findings are closely related with the findings of Saha (1997), Rajeswar and Masillamoni (2002), Samad *et al.,* (2007) and Khalil *et al.*, (2011).

In the ELISA test, it was found that the OD value of maternal antibody was 1.04±0.11 which was much higher than normal titer. But after BCRDV administration the OD value was not satisfactory (0.84±0.10) which may due to higher maternal antibody. Before experimental vaccination the OD value was 0.64±0.17. After experimental vaccination the OD value of all groups were (0.94±0.13, 0.90±0.117, 0.82±0.15, 0.76±0.11, 0.45±0.19) respectively. From the OD value, S/P value of five groups were calculated as 0.77, 0.74, 0.67, 0.65, 0.34 and antibody titres were determined as 2549.71, 2450.37, 2218.579, 2152.352, 1125.846 respectively. These finding were in agreement with Edison *et al.* (1982), Ernawati and Ibrahim (1984), Diaon *et al.,* (1998), Rahman *et al.,* (2004) and Molia *et al..* (2011).

Conclusions

In the present study velogenic isolates of NDV were isolated from suspected clinical cases of NDV. These isolates were confirmed as NDV by RT-PCR. The BHK-21 cell adapted ND killed vaccine developed experimentally here induced satisfactory level of antibody.

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