

*Full Length Research Paper*

# Biological characterization and determination of comparative efficacy of an inactivated Newcastle disease virus vaccine prepared from velogenic strain

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This study was designed to characterize a field isolate of Newcastle disease virus (NDV) to check its suitability as an inactivated vaccine and to determine the comparative efficacy of this inactivated NDV vaccine with conventional live vaccines, Baby Chick Ranikhet Disease Vaccine (BCRDV, F strain) and Ranikhet Disease Vaccine (RDV, Mukteswar strain). The field isolate of NDV was identified as velogenic strain based on the pathogenicity indices {mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI)}. Formalin precipitated inactivated NDV vaccine was prepared from the field isolate and adjuvanted with aluminium hydroxide. The comparative efficacy of three distinct vaccination schedules (Group A, inactivated vaccine three times only; group B, BCRDV followed by inactivated and group C, BCRDV followed by RDV) was then evaluated on the basis of haemagglutination inhibition (HI) antibody titre of sera samples collected at day 4, 27, 55 and 72 of post vaccinated chicks. The prepared inactivated vaccine with the field isolate induced satisfactory level of antibody following vaccination in chicken. Combined vaccination in chickens with live BCRDV followed by inactivated NDV vaccine induced better immune response than the live or inactivated NDV vaccine alone. The antibody titre though differed significantly ( $P < 0.05$ , group B versus group C at 55- and 72-days and group B versus group A at 72 days) in the chickens among the vaccinated groups, the protection rate was 100% in all groups following virulent challenge infection.

**Key words:** Newcastle disease virus, vaccine, velogenic, haemagglutination inhibition (HI), antibody.

## INTRODUCTION

Newcastle disease (ND) also known as Ranikhet disease (RD), is an acute infectious and contagious viral disease

of birds that has a worldwide distribution including Bangladesh and has a serious economic impact on poultry

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production (Biswas et al., 2009). The factors that affect the disease may be host, species, age, immune status, infection with other organisms and environmental stress (Cheville et al., 1972; Lancaster, 1981; Campbell, 1986). The disease is characterized by sudden appearance and rapid spread within the flock with high morbidity and mortality. It may cause 100% mortality in young chickens and 80-90% in adult chickens (Brandly, 1950; Chowdhury et al., 1982). Newcastle disease is endemic in Bangladesh with prevalence of viscerotropic velogenic strains (Chowdhury et al., 1982; Islam et al., 2003).

The etiologic agent of ND is a member of the avian paramyxoviruses (APMV), which belongs to the *Avulavirus* genus and *Paramyxoviridae* family of the order *Mononegavirales* (Abolnik et al., 2004; Pedersen et al., 2004). Paramyxoviruses isolated from avian species have been grouped into nine serotypes (APMV-1 to APMV-9), and Newcastle disease virus (NDV) is referred to as APMV-1 (Alexander, 2003). Strains of NDV are categorized into five pathotypes according to the clinical signs observed in infected chickens: (i) viscerotropic velogenic, (ii) neurotropic velogenic, (iii) mesogenic, (iv) lentogenic and (v) asymptomatic (Alexander, 2004).

Vaccination for ND is routinely practiced in countries where virulent strains of the NDV are endemic (Xiao et al., 2012). It has been practiced to control and prevent the ND from Bangladesh mainly by live NDV vaccine produced by Livestock Research Institute (LRI), Mohakhali, Dhaka, Government of the Peoples Republic of Bangladesh (two types of vaccines, BCRDV by F strain for baby chick and RDV by M strain for adult chicken). However, none of these strains were found to be used in controlling ND completely. Sometimes, reports on severe outbreak of ND are made even after vaccination of chicken with these live vaccines prepared from mesogenic and lentogenic (not local) strains and velogenic strain of NDV has been isolated from most of the outbreaks (Saha et al., 1998). The possible causes of outbreaks of ND in immunized flock were interfered by presence of maternal antibody and antigenic variation among the vaccine strains and field strains (Zhuo et al., 1998). Use of live vaccines in immuno-suppressed chicks is risky and conversely may produce disease. In Bangladesh, more than 70% rural households are involved in poultry keeping, where village vaccinators have been trying in going round villagers in their area vaccinating the family poultry and achieving high degree coverage. On the other hand, there are varieties of mode and means of transport and variation in maintenance of cooling system (4-8°C) at all stages of District, Thana, Union, Village and farm level in Bangladesh. This is one of the important reasons of vaccination failure causing economic loss to the farmers. To overcome these problems inactivated NDV vaccine is of growing interest and might be an efficient alternative which is already been practiced in many countries of the world (OIE, 2009). It has thus become important to isolate, identify

and characterize the NDV, selection of a suitable vaccine strain and preparation of prophylactic agent from the selected isolate that would not adversely affect the maternal immunity as live vaccine and can be used in day-old chicks (Box et al., 1976). The present study was undertaken to characterize a local field isolate of Newcastle disease virus and to determine the comparative efficacy of inactivated Newcastle disease virus vaccine prepared from velogenic strain with conventional live Newcastle disease virus vaccines.

## MATERIALS AND METHODS

### Study area and duration

The present research work was carried out during the period of January to May 2012 in Virology Laboratory and in the Experimental Poultry Sheds of the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh.

### NDV isolate

Newcastle disease virus isolate (NDV/MBD/8/2012) was obtained from the repository of the Virology Laboratory, Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh as per record of where the virus was isolated from a natural outbreak and was identified as NDV using polyclonal sera raised in chicken against reference strains of NDV.

### Reference NDV

Mukteswar, a Mesogenic strain of NDV was used as antigen in HI tests. Velogenic strain of NDV (NDV/DBD/1/2008) was used for challenge infection. The viruses were obtained from the repository of the virology laboratory, Department of Microbiology and Hygiene, Bangladesh Agricultural University (BAU), Mymensingh-2202.

### NDV live vaccine

Lyophilized baby chick Ranikhet disease vaccine (BCRDV, F-strain) and Ranikhet disease vaccine (RDV, Mukteswar) produced by Livestock Research Institute (LRI) Mohakhali, Dhaka, Department of Livestock Services (DLS), Government of the People's Republic of Bangladesh, were collected from the LRI, Dhaka.

### Experimental chicks

A total of forty eight (48) day old Layer BB300 chicks were obtained from the Phenix Hatchery Ltd. Gazipur. The chicks were reared with feed and water *ad libitum* for 12 weeks maintaining strict biosecurity and in a well-ventilated experimental poultry shed of the Department of Microbiology and Hygiene, BAU, Mymensingh according to university animal care and use guidelines.

### Pathogenicity indices of field isolate of NDV

The mean death time with the minimum lethal dose (MDT/MLD) was determined with 9-day-old chicken embryos following the procedure described by Hanson and Brandly (1955). The intracerebral pathogenicity index (ICPI) with day-old chicks was

determined following the method described in OIE (2009). The intravenous pathogenicity index (IVPI) with 6-week-old chickens was determined following the method described by Alexander (1998).

#### **Titration and inactivation of the field isolate of NDV**

The infectivity titre of NDV was determined by inoculating serial 10-fold dilutions ( $10^{-1}$  to  $10^{-10}$ ) of virus in the form of allantoic fluids, into embryonated chicken egg (ECE). The end point titre was expressed as 50% embryo lethal dose (ELD<sub>50</sub>) per ml as calculated by the method described by Reed and Muench (1938). Infective allantoic fluid (AF) containing field isolate of NDV having ELD<sub>50</sub>  $10^{8.7}/1$  ml of AF was inactivated by treating with formalin at 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 allantoic fluids.

#### **Preparation of inactivated NDV vaccine**

The inactivated NDV vaccine was prepared following the method described by Tizard (1996). Briefly, after proper inactivation of the virus, aluminium hydroxide (AH) was added as adjuvant at the rate of 0.6 ml/1 ml of properly inactivated NDV vaccine (According to LRI) and was mixed properly by vortex machine.

#### **Sterility and safety test of the inactivated NDV vaccine**

The sterility and safety test of the inactivated NDV vaccine was done according to the OIE (2009) by using blood agar media and six 5-day-old chicks, respectively.

#### **Experimental design**

A total of forty eight (48) day-old Layer BB300 chicks were collected from the Phenix Hatchery Ltd. and used for this experiment. The chicks were divided into four (4) groups each containing 12 chicks viz group A, B, C and D. Chicks of different groups were vaccinated following three distinct vaccination schedules. Chicks of group A were immunized thrice with inactivated NDV vaccine through intramuscular (i/m) route at 5-, 28- and 56-days of age at the dose rate of 0.25, 0.5 and 1 ml/ bird, respectively. Chicks of group B were immunized with BCRDV through intraocular (i/o) route at 5 days of age at dose rate of 1 drop/bird and with inactivated NDV vaccine through i/m route at 28- and 56-days of age at the dose rate of 0.5 ml and 1 ml/bird respectively. Chicks of group C were immunized with BCRDV through i/o route at 5- and 28-days of age at dose rate of 1 drop/bird and with RDV through i/m route at 56 days of age at the dose rate of 1 ml/bird. Chicks of group D were kept as unvaccinated control. The comparative efficacy of the three vaccination schedule was then evaluated by measuring HI antibody titre of sera samples collected at day 4, 27, 55 and 72 of chicks.

#### **Haemagglutination inhibition (HI) test**

The HI test was performed following the method described by Anon (1971) to determine the HI antibody titre of the sera samples collected from all groups of vaccinated chickens at different intervals following administration of ND vaccines. The HI titre of sera samples of control group of chicks was also determined to measure the maternal antibody and its persistence. The test was conducted by using constant 4 HA unit antigen and decreasing serum method (beta-procedure). Briefly, 50 µl PBS was taken in all

the wells of 96 well plate. Then 50 µl of heat inactivated (56°C for 30 min) serum was taken in the first well of the respective row and a two-fold serial dilution of the serum was prepared. Then, 50 µl of antigen suspension containing 4 HA units was added into all well except the last well of the row and mixed thoroughly. The last well of respective row was kept as control for respective sample. The serum antigen mixture was then incubated for 30 min at room temperature. Then 50 µl of 0.5% cRBC suspension was added into all well. Then the mixture was again kept at room temperature for 45 to 60 min. A compact mass of sediment cells covering the bottom of the plate was considered as positive for HI. Serum end point was determined as the highest dilution of serum, which inhibited the agglutination of the RBC in the test. The HI titre of each serum corresponded to reciprocal of highest original dilution of serum inhibiting agglutination of cRBC completely.

#### **Protection test**

Sixteen days after final immunization, the chickens were challenged through intranasal (i/n) route with 0.1 ml of allantoic fluid containing 2ELD<sub>50</sub> of NDV which correspond to about 100% mortality in chickens of 10 weeks of age (Sarkar et al., 2012).

#### **Analysis of data**

All data (HI antibody titre) were expressed as mean  $\pm$  SE and difference serum antibody titres among the groups of chickens were compared using one-way ANOVA. Statistical analysis was performed using SPSS software version 17 and significance level was set at  $P \leq 0.05$ . Survival rate among the different groups after challenge were analyzed by Mantel-Cox log rank test.

## **RESULTS**

### **Pathogenicity indices**

The results of the mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) of the field isolate of NDV were found to be 57.6, 1.81 and 2.69 h, respectively and values indicate the isolate is velogenic.

### **Sterility and safety test of the inactivated NDV vaccine**

The inactivated NDV vaccine was found to be sterile as no bacterial growth was observed in inoculated blood agar media incubated at 37°C for 48 h. Following inoculation in naive chicken, the inactivated NDV vaccine was found safe as the chick showed no significant effects after administering double dose of prepared inactivated NDV vaccine through intramuscular (i/m) route and subcutaneous (s/c) route.

### **HI antibody titre of vaccinated and unvaccinated control chickens**

The HI antibody titres (mean  $\pm$ SE) of vaccinated chickens

**Table 1.** Comparative HI antibody titer of chickens of vaccinated groups and unvaccinated control, Group D.

Chickens groups	Age of chickens			
	Day 04	Day 27	Day 55	Day 72
	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE
Group A (Vaccinated with inactivated NDV vaccine)	77.33 $\pm$ 9.20	85.33 $\pm$ 9.09	181.33 $\pm$ 19.03	469.33 $\pm$ 28.77
Group B (Vaccinated with BCRDV and inactivated NDV vaccine)	69.33 $\pm$ 8.66	90.67 $\pm$ 9.51	213.33 $\pm$ 18.19	597.33 $\pm$ 57.53
Group C (Vaccinated with BCRDV and RDV)	72.00 $\pm$ 8.00	96.00 $\pm$ 9.65	160.00 $\pm$ 16.71	490.67 $\pm$ 21.33
Group D (Unvaccinated control)	85.33 $\pm$ 9.09	50.67 $\pm$ 4.76	26.67 $\pm$ 2.27	4.33 $\pm$ 0.33

Chickens of group A were immunized thrice with inactivated NDV vaccine through i/m route at 5-, 28- and 56-days of age. Chickens of group B were immunized with BCRDV through i/o route at 5 days of age and with inactivated NDV vaccine through i/m route at 28- and 56-days of age. Chickens of group C were immunized with BCRDV through i/o route at 5- and 28-days of age and with RDV through i/m route at 56 days of age. Chickens of group D were kept as unvaccinated control. Serum was collected from chickens of all vaccinated groups (Group A, B and C) and unvaccinated control group (Group D) at 4-, 27-, 55- and 72-days of age. Serum antibody titers of chickens against different NDV vaccines and control birds were determined by HI test. The table shows the mean  $\pm$  SE values of serum HI antibody titre (n=12 chickens/group), where SE = Standard error, HI = Haemagglutination inhibition.

and unvaccinated control group are presented in Table 1. It was observed that serum antibody titre of chickens of all vaccinated group (Group A, B and C) was gradually increased up to 72 days of birds following primary, secondary and tertiary vaccination (Table 1). On the other hand, serum antibody titre (Maternal antibody titres) of chickens of unvaccinated control group D decreased gradually (Table 1).

### Protection test

The results of the protection test is presented in Figure 4, All the chickens exhibited no signs of illness and survived after challenge infection throughout the period of observation of 10 days. On the other hand, all the control chickens challenged at the same day started to show typical clinical signs of ND such as gasping, greenish-dark watery diarrhoea, drowsiness, dropping of wings etc from 2<sup>nd</sup> day onward and started to die from day 3 onwards. Dead birds showed typical postmortem lesions on ND such as hemorrhage in the trachea, proventriculus and ulceration of intestinal mucosa. Based on the protection test, it appeared that chickens of vaccinated groups (Groups A, B and C) conferred 100% protection following virulent challenge (Figure 4).

### DISCUSSION

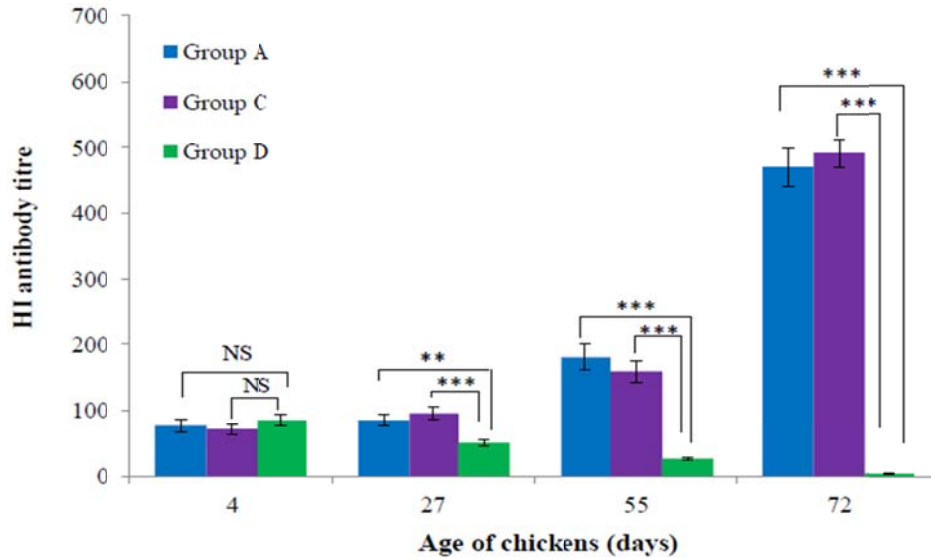
The results of the MDT, ICPI and IVPI of the field isolate of NDV were found to be 57.6 h, 1.81 and 2.69, respectively. These findings suggest that the isolate belong to velogenic group and correlated with the criteria mentioned by several investigators (Adi et al., 2010;

Munir et al., 2012; Courtney et al., 2013).

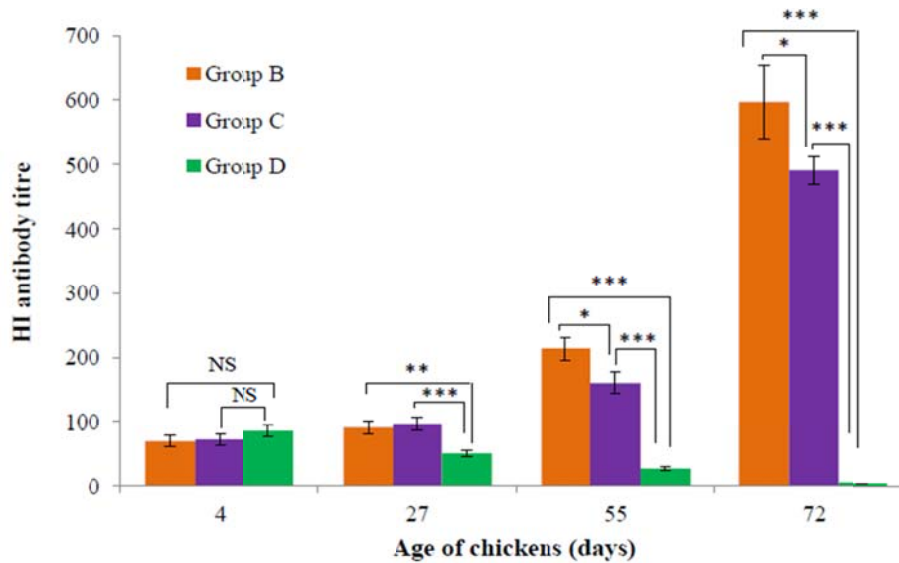
The mean  $\pm$  SE of HI antibody titre of chickens of group C at 27- (22 days after primary vaccination) and 55-days (27 days after secondary vaccination) of age (Table 1 and Figure 1) showed that serum antibody level of chickens (Group C) following primary vaccination did not show any impetuous production 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 (Sarkar et al., 2012; Cornax et al., 2012; Kapczynski et al., 2012). On the other hand, HI antibody titre of chickens after secondary vaccination increased significantly ( $P < 0.01$ , day 27 versus day 55) and similar finding were reported by other investigators (Sarkar et al., 2012; Kafi et al., 2003; Shuaib et al., 2003). The mean  $\pm$ SE of HI titre at 72 days of age (16 days after tertiary vaccination) of chickens of group C (vaccinated with RDV) indicated that serum antibody level of chickens (Group C) was increased significantly ( $P < 0.001$ , day 55 versus day 72) following tertiary vaccination at 56 days of age (Sarkar et al., 2012; Banu et al., 2009; Chowdhury et al., 1981).

The mean  $\pm$ SE of HI antibody titres of chickens of group A (Table 1 and Figure 1) vaccinated thrice with inactivated NDV vaccine through i/m route at 5-, 28- and 56-days of age were increased from day 4 to day 72 of age and the titre was increased significantly at 55- and 72-days ( $P < 0.001$ , day 27 versus day 55 and day 55 versus day 72) of age. These HI antibody titres of chickens of group A is mostly similar to HI antibody titre of the chickens of group C (Fan et al., 2012; Iqbal et al., 2003 and Rajeswar and Masillamoni, 2002).

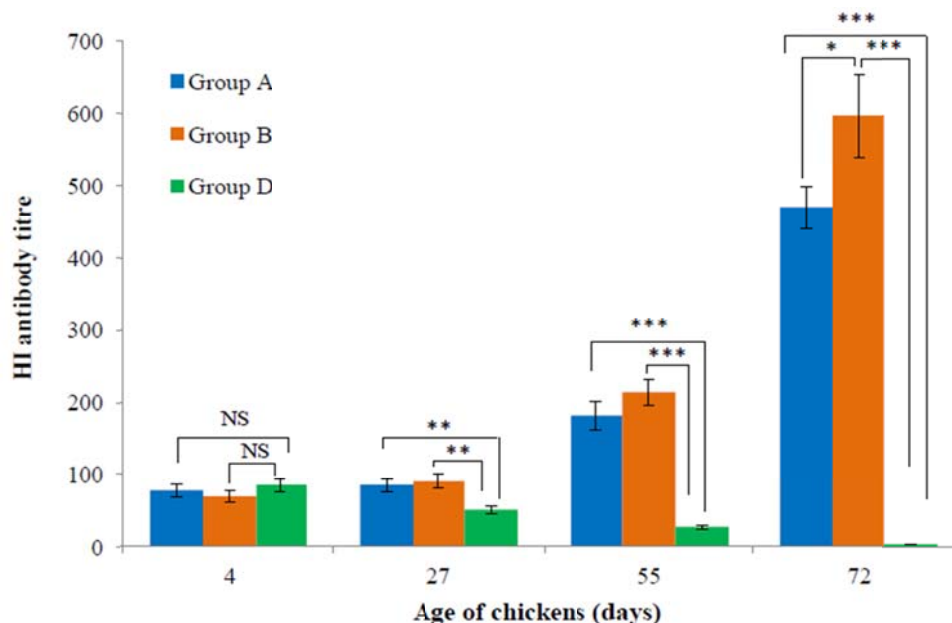
The mean  $\pm$ SE of HI antibody titres of chickens of group B (Table 1 and Figure 2) vaccinated once with



**Figure 1.** Comparative HI antibody titre of vaccinated groups (A and C) and unvaccinated control (D). Chickens of group A were immunized thrice with inactivated NDV vaccine through i/m route at 5-, 28- and 56-days of age. Chickens of group C were immunized with BCRDV through i/o route at 5- and 28-days of age and with RDV through i/m route at 56 days of age. Chickens of group D were kept as unvaccinated control. Serum was collected from chickens of vaccinated groups (Group A and C) and unvaccinated control group (Group D) at 4-, 27-, 55- and 72-days of age. Serum antibody titers of chickens against different NDV vaccines and control birds were determined by HI test. The graph shows the mean  $\pm$  SE values of serum HI antibody titre (n=12 chickens/group). NS = Non significant, \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001 by one-way ANOVA.



**Figure 2.** Comparative HI antibody titre of vaccinated groups (B and C) and unvaccinated control (D). Chickens of group B were immunized with BCRDV through i/o route at 5 days of age and with inactivated NDV vaccine through i/m route at 28- and 56-days of age. Chickens of group C were immunized with BCRDV through i/o route at 5- and 28-days of age and with RDV through i/m route at 56 days of age. Chickens of group D were kept as unvaccinated control. Serum was collected from chickens of vaccinated groups (Group B and C) and unvaccinated control group (Group D) at 4-, 27-, 55- and 72-days of age. Serum antibody titers of chickens against different NDV vaccines and control birds were determined by HI test. The graph shows the mean  $\pm$  SE values of serum HI antibody titre (n=12 chickens/group). NS = Non significant, \* $P$  < 0.05, \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001 by one-way ANOVA.



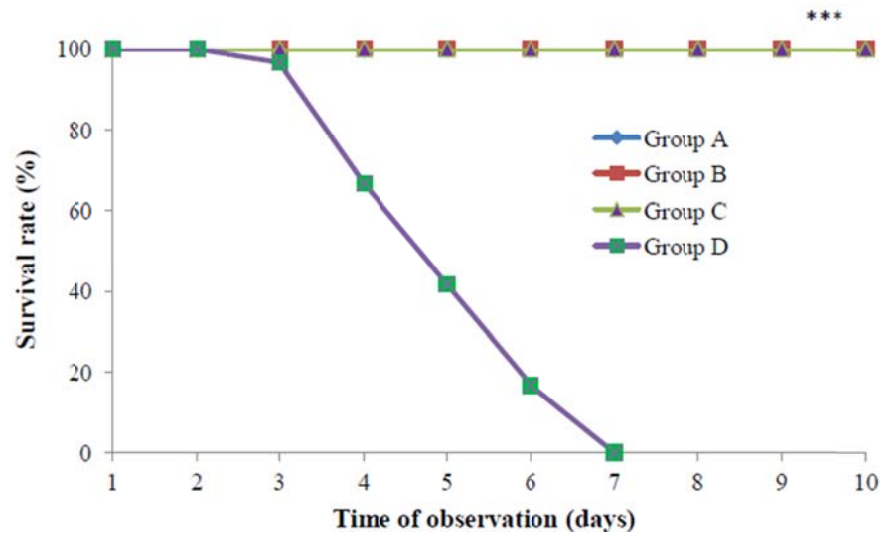
**Figure 3.** Comparative HI antibody titre of vaccinated groups (A and B) and of unvaccinated control (Group D). Chickens of group A were immunized thrice with inactivated NDV vaccine through i/m route at 5-, 28- and 56-days of age. Chickens of group B were immunized with BCRDV through i/o route at 5 days of age and with inactivated NDV vaccine through i/m route at 28- and 56-days of age. Chickens of group D were kept as unvaccinated control. Serum was collected from chickens of vaccinated groups (Group A and B) and unvaccinated control group (Group D) at 4-, 27-, 55- and 72-days of age. Serum antibody titers of chickens against different NDV vaccines and control birds were determined by HI test. The graph shows the mean  $\pm$  SE values of serum HI antibody titre (n=12 chickens/group). NS =Non significant, \* $P$  < 0.05, \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001 by one-way ANOVA.

BCRDV at 5 days of age through i/o route and twice with inactivated NDV vaccine through i/m route at 28 and 56 days were increased from day 4 to day 72 of age and the titre was increased significantly at 55 ( $P$  < 0.01, day 27 versus 55 day) and 72 days ( $P$  < 0.001, day 55 versus day 72) of age. But the HI titres of the chickens of group B were significantly higher ( $P$  < 0.05) than the chickens of group A (vaccinated thrice with inactivated NDV vaccine) at 72 days of age and group C (vaccinated twice with BCRDV and once with RDV) at both 55- and 72-days of age (Figures 1 and 3). This result strongly supported the previous results of several scientists (Barbour et al., 2011; Wanasawaeng et al., 2009; Kafi et al., 2003). This result indicated that chickens vaccinated primarily with live NDV vaccine followed by inactivated NDV vaccines produce strong immune response.

To investigate the persistence of maternal antibody for any possible interference or hindrance to the vaccines that might have occurred, sera samples obtained from the chickens of unvaccinated control group D at 4-, 27-, 55- and 72-days of age were analyzed. It was observed that the HI antibody titres of chickens of unvaccinated control group gradually decreased from day 4 to day 76 of age (Sarker et al., 2012; Kai et al., 2012). Two live vaccines (BCRDV and RDV) are being used to control

Newcastle disease in Bangladesh. Chicks having high maternal antibody titer interfered with the immune response when vaccinated with BCRDV (Sarker et al., 2012) so live vaccine (BCRDV) followed by live vaccine (RDV) do not induce strong antibody response. So farmers are using imported killed NDV vaccines which are expensive and urging to develop a killed vaccine which might be economic and save the growing poultry industry from Newcastle disease in Bangladesh. Inactivated vaccines were not imported, except farms rearing parent stock commercially. The benefit of using inactivated vaccine followed by live vaccine result in high and long lasting immune response. With the increase of commercial poultry farms in Bangladesh the demand for inactivated vaccine is on the rise as well.

Chickens of all vaccinated (Group A, B and C) and unvaccinated group were challenged at 72 days of age (16 days after tertiary vaccination) with virulent field isolate of NDV. The chickens (Groups C) vaccinated with conventional live vaccines (BCRDV and RDV) provided 100% protection (Sarker et al., 2012; Cornax et al., 2012). The chickens vaccinated thrice with inactivated NDV vaccine (Groups A) and vaccinated with BCRDV followed by inactivated NDV vaccine (Group B) also conferred 100% protection, similar to chickens of group C



**Figure 4.** Survival rate of chickens of vaccinated groups (A, B and C) and unvaccinated group (D). Chickens of group A were immunized thrice with inactivated NDV vaccine through i/m route at 5-, 28- and 56-days of age. Chickens of group B were immunized with BCRDV through i/o route at 5 days of age and with inactivated NDV vaccine through i/m route at 28- and 56-days of age. Chickens of group C were immunized with BCRDV through i/o route at 5- and 28-days of age and with RDV through i/m route at 56 days of age. Chickens of group D were kept as unvaccinated control. Sixteen days (72 days of age) after final immunization (Tertiary vaccination) of chickens of all vaccinated group (Group A, B and C) and chickens of unvaccinated control group (Group D) were challenged with virulent field isolate of NDV at the rate of 0.1ml of 2ELD<sub>50</sub> per bird intranasally and survival rate of chickens was observed for subsequent 10 days. (n=12 chickens/group). \*\*\**P* <0.001 by Mantel-Cox log rank test.

(Kai et al., 2012; Diaon et al., 1998). On the other hand, chickens of unvaccinated control group (Group D) had no protection capacity following virulent challenge infection (Figure 4).

From the above discussion it may be summarized that the field isolate of the NDV was velogenic and prepared inactivated vaccine from this isolate induced satisfactory level of antibody after vaccination in chicken. Combined vaccination in chickens with live (BCDRV) vaccine followed by inactivated NDV vaccine induced better immune response than the live or inactivated NDV vaccine alone. Although the antibody titre differs significantly (*P* <0.05, group B versus group C at 55- and 72- days and group B versus group A at 72 days) in the chickens among the vaccinated groups, the protection rate was 100% in all groups following virulent challenge infection.

## Conclusions

Inactivated NDV vaccine prepared from velogenic strain of NDV provided similar protection as live NDV vaccine. So, inactivated NDV vaccine may be used for the prevention and control of ND in Bangladesh and vaccination program against ND should be rescheduled with live BCRDV vaccine followed by inactivated NDV vaccine.

## Conflict of Interests

The authors have not declared any conflict of interests.

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