EVALUATION OF CROSS PROTECTION AGAINST AVIAN INFLUENZA VIRUS (AIV) AND NEWCASTLE DISEASE VIRUS (NDV) IN BROILER CHICKENS AFTER VACCINATION IN PAKISTAN

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ABSTRACT

Newcastle Disease is still endemic in Pakistan's poultry industry and responsible for great economic losses and high mortalities. Avian Influenza Virus has been involved in production losses in past few years, with or without the association of Newcastle Disease in the field. Therefore, present study was planned to evaluate the cross protection between Avian Influenza Virus and Newcastle Disease Virus. Local field isolates for both viruses were collected from recent outbreaks and used for inactivated vaccine formation. Results revealed that geometric mean titer values assessed up to 6 ($\log_2$) or above, provided excellent protection to chickens when challenged with viscerotropic velogenic form of Newcastle Disease Virus (vvNDV). Groups vaccinated with inactivated Newcastle Disease vaccine and bivalent vaccine (containing Newcastle Disease Virus and Avian Influenza Virus) provided 90 to 100% protection following challenge with vvNDV. Oil emulsified vaccine provided excellent protection using local field isolate against Newcastle Disease. Moreover, inactivated Avian Influenza vaccine produced 60% cross protection using local field isolate when challenged with vvNDV. Regression analysis showed significant (P< 0.01) correlation between titers against Avian Influenza versus Newcastle Disease, though the group was only vaccinated with Avian Influenza vaccine. Moreover, the group vaccinated with Newcastle Disease vaccine produced titers against Newcastle Disease as well as Avian Influenza despite the group was not vaccinated against Avian Influenza Virus and correlation among them was found highly significant (P< 0.001).

Key words: Newcastle disease, Avian influenza virus, Cross protection, Inactivated vaccine.

INTRODUCTION

Livestock sector is developing day by day with growth rate 3.70% and in its major share is by poultry sector (10.4%). Pakistan poultry sector is very much ahead in getting international standard production within the given circumstances. Newcastle disease (ND) is the top ranking disease of rural poultry in Pakistan (Khan *et al.*, 2011). Moreover, ND problems were recorded during last winter outbreaks (November 2011 to march 2012) from all over Punjab areas of Pakistan (Ali *et al.*, 2014). It caused unprecedented mortalities in broilers and production losses occurred both in layers as well as in breeders. Although ND is a long known disease it is still not possible to control it. In spite of the use of massive ND vaccines, the virus still breaks through protection and causes outbreaks in broiler farming. Published data proved that amino acids sequence at cleavage site is responsible for pathogenicity of ND virus, which determine the range of protease enzymes that cut the cleavage site and making fusion protein activated (Smietanka *et al.*, 2006). Recent studies have revealed that existing AIV is continuously mutating. Based on the field experience of AIV (H_9N_2) infection, it appears that this infection has very close clinical signs and symptoms with those caused by mild strains of ND (Siddique *et al.*, 2008). Their surface is covered by haemagglutinin (HA) and neuraminidase (NA) glycoprotein projections (Numan *et al.,* 2005). Influenza type A virus infection in chickens occurs in two forms, Highly pathogenic avian influenza (HPAI) also known as "Fowl Plague", with mortality up to 100%, and low pathogenic avian influenza (LPAI) usually causes mild clinical signs with slight drop in egg production in layer case (Alexander, 2003; Siddique *et al.*, 2008; AL-Barwary *et al.*, 2012). HPAI derivatives originated from LPAI (H_5) by re-assortment in a combination of in vitro and in vivo experimental systems. More often, wild birds transmit LPAI viruses to poultry, and these viruses then mutate to become HPAI viruses while they are circulating in poultry flocks (Siddique *et al.*, 2008). The goal of the present research work was to evaluate an immune status of broiler chickens with and without vaccination against NDV and AIV separately and in combination and protection/cross protection when challenged with NDV.

MATERIALS AND METHODS

Newcastle disease virus and Avian Influenza virus (local field isolates) were used for the vaccine

preparation in the experimental trial. These viruses were isolated from the recent outbreaks and confirmed by post-mortem examination as well as by molecular method (RT-PCR). Biological titrations were done by making tenfold dilutions of each virus and inoculating each dilution into five eggs. Egg Infective Dose 50 (EID_{50}) for NDV and AIV was found $10^{9.16}$ EID_{50} and $10^{9.46}$ EID_{50} per ml respectively, calculated following Spearman and Karber method (1974). Embryonated chicken eggs of 9 day-old were inoculated with field isolate virus at the rate 0.2ml/egg following Ali *et al.* (2014). Harvested fluid (Aamnio Allantoic Fluid, AAF) centrifuged at 4,000 rpm (Hettich Zentrifugen, Germany) for 10 minutes and supernatant was collected for inactivation. Formalin (37.5%) with dose rate 0.05% was used for inactivation. Thimerosal was used with dose rate 0.01% to avoid bacterial and fungal growth in the AAF. The interaction time of formalin and AAF was provided 24 hours at 37°C in an orbital shaking incubator (Model # PA-H-42/250). Inactivation was confirmed by inoculating inactivated virus into embryonated eggs up to three consecutive passages. Oil based vaccine was prepared by emulsifying inactivated virus in Montanide oil (ISA 70M VG) (Seppic, France). Inactivated virus was added drop wise in Montanide oil and mixed with homogenizer (T25 basic ULTRA-TURRAX, IKA (Germany)) continuously and stable white (water in oil) emulsion was prepared.

Experimental Flock: Study was conducted on post hatched (n=200) day-old broiler chicks (*Gallus domesticus*), having an average body weight 40gm/chick, during April 2013 to June 2013. Rearing of chicks was arranged in a semi-controlled shed located 23Km away from Muridke (Northern Punjab) Pakistan. The parameters like feeding, medication, vaccination and environmental conditions were adopted similar to the field practices. After 4 days of acclimatization, birds were divided randomly into five groups. Group **A** (n=40)**, B** (n=40)**, C** (n=40), **D** (n=40) and Group **E** (n=40) was kept as a control (Table-1).

Serology: Blood samples were collected from 5% birds randomly prior to vaccination and maternal immunity was assessed at 4th day of age. Ten birds from each group (A, B, C, D and E) on 14th, 21st, 28th, 35th and 42nd day respectively were selected randomly and blood samples were collected. Sera were drawn from each sample labeled and stored at -20C till further use. Haemagglutinition Inhibition titers were determined and geometric mean titer (GMT) values were calculated following Brugh, 1978.

Challenge Virus: Vaccinated birds were challenged at day 23rd and 37th post vaccination with NDV field isolate (CK/NDV/KSUR/PK/2011/55) characterized as velogenic at Quality Operation laboratory, University of Veterinary and Animal Sciences Lahore. The virus

challenge dose was 10,000 units of CLD_{50} ($10^{4.5}$) /ml/bird calculated following Spearman and Karber method (1974). Birds were challenged by eye drop method and kept under observation for 15 days for development of any abnormality or mortality.

Data Analysis: In order to data analysis, titers and GMT values were calculated following Brugh, (1978). Data analysis was performed using statistical package SPSS 16.0 software. Correlation coefficient was calculated to check the significance level of relationship between the given variables. Results were interpreted following Zar, (1996).

RESULTS AND DISCUSSION

Maternal antibody titers at the age of 4th day were found GMT (log_2) 4.1 and 3.3 against ND and AI virus respectively, evaluated by haemagglutinition inhibition (HI) test. In group A, titers recorded at peak level after 23rd days post vaccination (PV). Furthermore, the titers against AIV were also recorded that remained up to 42nd day of age, though the group was not vaccinated against (AIV) (Fig-I). Statistically, highly significant correlation (P<0.001) among titers of NDV versus AIV was found in group A (Table-2). While in group B titers were at peak level at the age of 28th day (titers 5.5). However, a decline after one week was also recorded as 4.4 less 1.1 (log_2) and remained almost same up to 42nd day of age. While control group showed almost same HI titer against NDV; varying from 3 to 3.8 during the entire duration of the trial. Serological assessment revealed that Group C not only produced high titers (0.6 to 7.5 log_2) against AIV but also produced titers against NDV though the group was not exposed to ND virus. The titers produced against NDV were recorded 6.4 after having a booster dose of killed AIV vaccine at 18th day and remained 5.3 more or less up to marketing age (Fig-II). Furthermore, significant correlation (P< 0.01) was observed between titers of AIV versus NDV (Table-3), as well as provided 60% cross protection when challenged with virulent form of NDV (Table-4). Group D produced titers against NDV 3.6 (log_2) and two fold increase a week later, while titers against AIV linearly increased ranged from 1.6 to 7.3 (log_2). When challenged with vvNDV, 100% efficacy was recorded. The control group (E) showed 100% mortality, confirms the virulence of the challenge dose (Table-4). Vaccination interfere with the maternal antibodies, similar observation were recorded in the trial at 9 day PV against ND and AI occurred due to neutralization of the vaccine virus by circulating maternal antibodies (Tizard, 2004). However, interference to maternal immunity due to live vaccine as a booster dose (Group B) after ND inactivated vaccine reduced the titers from 2.1 to 0.5. The reason of this decline was explained by Prescott (2003) as the live attenuated ND vaccine

produce immune suppression and chickens become susceptible to disease. A booster dose of ND killed vaccine produced effective and long term immunity, retained up to 42 days (marketing age) also reported by World Organization for Animal Health, (2012). Existence of AI titers not related to the vaccination against AI, however, the flocks were regularly vaccinated against ND (unpublished data). Our observations confirm the unpublished data as similar observations were recorded in group A and GMT values against AI ranged from 1.6 to 7.0 with a linear increase from 14th day of age to 42nd day though group was only vaccinated against ND. Furthermore, an increase of AI titers (Group A) might be due to the factor that various serotypes AI particularly H5, H7, and H9 are circulating in our environment since 1995 and they are naturally priming/adapting in this hemisphere (Unpublished data). Interference to maternal immunity with killed vaccine was found lowest as it reduced the titers from 4.1 to 3.6. However, the use of live attenuated ND vaccine as a booster dose enhance an increase of two fold the immunity almost double but rapid decline in the HI titers within a week was recorded and remained almost same up to 42 days of age. Erganis and Ucan, (2003) showed that the antibody titers to ND in a flock vaccinated with live vaccines decreased more rapidly than those of flocks vaccinated by inactivated antigen. The factors contributing in ND outbreaks like poor quality of vaccine and lack of a cold chain system cannot be overruled even though stringent vaccination schedule against ND (Abolnik *et al.,* 2004; Bogoyavlenskiy *et al.,* 2009; Hassan *et al.*, 2010; Ke *et al.,* 2010). Antigenic variance might be another cause among circulating velogenic field strain of NDV and vaccinal virus (Kapczynski and King, 2005; Qin *et al.,* 2008; Van-Boven *et al.,* 2008; Miller *et al.*, 2009; Hu *et al.,* 2009). Even with ND vaccination, outbreaks are still happening which indicate mutation, which might be occurring in the virus structure (Ahmed *et al.*, 2009). Moreover, vaccine will provide excellent protection if field virus and vaccine virus are antigenically same, as mutation in viral protein will reduce its efficacy. Therefore, it is recommended to use the recent indigenous isolate for vaccine manufacturing. The present study findings are in support of authors mentioned before, but our results do not support experts expressed published reports (Jeon *et al.,* 2008; Dortmans *et al.,* 2012), who reported that ND outbreaks are not due to antigenic variation. Furthermore, low level of immunity or concurrent infection with immunosuppressive pathogens in flock may be responsible of outbreaks due to improper vaccination strategies. Moreover, study on NDV isolates collected from Karachi revealed the existence of two genotypes (VI and VII) in Pakistan (Khan *et al.*, 2010). Mismatched genotype may be another reason of vaccine failure as commercially available vaccines fall in genotype II while circulating field virus fall in genotype VII (unpublished data). Similar findings also reported by Munir *et al.* (2012) that genotype VII primarily present in Asian countries (e.g. Korea, Taiwan and China) since 1980. Wild birds play vital role in virus spreading from country to country, therefore, different genotypes may co-circulate in a zone and cause disease (Mase *et al.* 2009). The reasons of these outbreaks was explained by Abolnik *et al.* (2004) that current ND vaccines have been used for more than 50 years, belonged phylogenetically to genotypes (I and II) as viruses isolated in the 1940s. Additionally, antigenic variation occurred in last two decades that cause ND outbreaks. In United States predominantly isolated virus from outbreaks belong to genotype V while in Asia, Africa and Europe it belongs to genotype VI and VII (Abolnik *et al.*, 2004). When we used AI killed vaccine an interesting finding was recorded that it produced 60% cross protection against ND, even the group was not vaccinated with ND killed vaccine but challenged with vvNDV field isolate, shows that for somehow there was some association in protecting these two viruses in the field. Furthermore, regression analysis showed a significant (P< 0.01) relationship among HI titers of AI and ND, though the group was only vaccinated against AI. This shows some correlation in the viral multiplication between AI and ND antigens that lead to trigger the immune system of the chicken. The results reveal that there is a similarity in group C and group A, because both of them giving long term cross immunogenicity to each other without vaccination. The best linear relationship was observed in the group vaccinated with bivalent (ND+AI) vaccine, further confirmed by the statistical analysis as well as 100% protection against challenged with vvNDV field strain. As reported by Tian *et al.* (2005) HI titers 4 ($\log_2$) or more is enough to provide complete protection to broiler after challenge. Contrary to above work, this experimental trial revealed that GMT values of 6 ($\log_2$) or above provided good protection to chickens following challenge with vvNDV. In Pakistan, presently, inactivated ND vaccines are being imported and it consumes a reasonable foreign exchange. However, use of locally produced inactivated vaccines will ultimately reduce the high economic losses due to import of ND vaccines used in broiler industry in Pakistan. Therefore, present study of an NDV indigenous isolate in Pakistan can serve as a basis for future vaccine design. The study high lights the importance of indigenous isolate in the manufacturing of the vaccine to counter-epidemic in the country.

Table-1. Experimental flock with vaccination schedule of different groups

Day of vaccination	Group A	Group B	Group C	Group D	Group E
5th day	ND killed vaccine (S/C)	ND killed vaccine (S/C)	AI killed vaccine (S/C)	Bivalent killed vaccine (S/C)	
7th day	ND-IB* vaccine (D/W)	ND-IB* vaccine (D/W)		ND-IB* vaccine (D/W)	
10th day	IBD (D/W)	IBD (D/W)	IBD (D/W)	IBD (D/W)	IBD (D/W)
18th day (Booster dose)	ND killed vaccine (S/C)	ND-clone (Live) spray method	AI killed vaccine (S/C)	Bivalent killed vaccine (S/C)	

*Commercial Vaccine ND+IB and ND-clone vaccine   S/C = Subcutaneously, D/W= Drinking Water

Table-2. GMT values of NDV versus AIV when vaccinated with NDV inactivated vaccine
Statistical parameters of various relationships involving GMT values of NDV versus AIV, probabilities (p), n = 30 in each case.

Relationships	Correlation coefficient (r)	Intercept (a)	Regression coefficient (b)	Standard error of b (S.E.)
GMT of NDV (inactivated NDV vaccine), (x) GMT of AIV (inactivated NDV vaccine), (y)	0.6490***	0.2484	0.7283	1.6880

***P < 0.001   Highly significant

Table-3. GMT values of AIV versus NDV when vaccinated with AIV inactivated vaccine
Statistical parameters of various relationships involving GMT values of AIV versus NDV, probabilities (p), n = 30 in each case.

Relationships	Correlation coefficient (r)	Intercept (a)	Regression coefficient (b)	Standard error of b (S.E.)
GMT of NDV (inactivated AIV vaccine), (x) GMT of AIV (inactivated AIV vaccine), (y)	0.5347**	2.3293	0.4321	1.8080

**P < 0.01   Significant

Table-4. Percentage protection in different groups challeneged with vvNDV virus at 28th and 42nd day

Group	28th day		42nd day	
	Mortality %	Protection %	Mortality %	Protection %
A	10	90	0	100
B	10	90	20	80
C	40	60	40	60
D	0	100	0	100
E	100	0	100	0
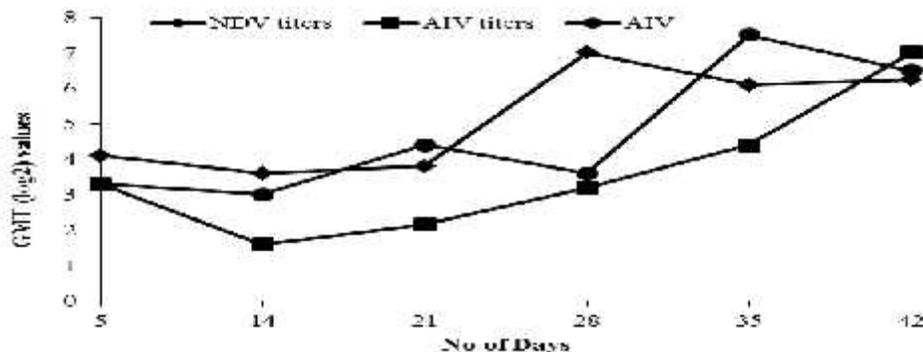
Fig-1. Correlation of cross immunogenicity of Geometric Mean Titers (GMT) log$_2$ of NDV versus AIV after NDV killed vaccination
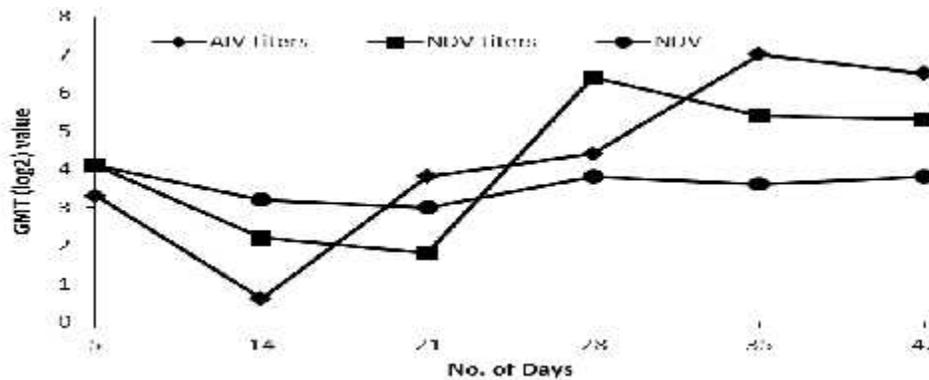
Fig-2.   Correlation of cross immunogenicity of Geometric Mean Titers (GMT) log$_2$ of AIV versus NDV after AIV killed vaccination.

Conclusion: The results of the current research revealed that 60% cross protection produced when birds vaccinated with killed AIV vaccine and challenged with ND virus. Moreover, regression analysis revealed high significant (P<0.001) correlation among HI titers against ND and AI. This showed structural similarity of surface epitopes among both of the viruses. Moreover, high and long term immunogenicity produced of the inactivated ND vaccine using indigenous isolate and its implementation under local field conditions. Bivalent vaccine (NDV + AIV) is recommended for an effective ND control program in endemic areas of Pakistan.

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