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ISOLATION, CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS AND COMPARITIVE EFFICACY OF DIFFERENT VACCINE REGIMES IN BROILER BIRDS

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ABSTRACT

Newcastle disease is a highly contagious disease of poultry and is cause of huge economic losses during 2011-2012 in Pakistan. Regardless of vaccination the disease has caused considerable mortalities in all types of poultry. In this study samples were taken from suspected flocks showing signs and symptoms of the disease. Isolation was done by chicken embryo inoculation and identification of the virus was done by Haemagglutination (HA) and Haemagglutination Inhibition (HI) assay. Presence of the virus was confirmed by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). All of the four virus isolates were found velogenic when pathotyped on the basis of Mean Death Time (MDT) and Intra Cerebral Pathogenicity Index (ICPI). Killed Vaccine was prepared from Chicken/Broiler /Attock /2012 having maximum pathogenicity. Evaluation of the vaccine was done in randomly divided seven groups of broilers each having 42birds.One group was kept as control. These groups were vaccinated with different vaccines and different vaccine schedules. There was non-significant difference in HI log 2 titers of different groups. Vaccinated group in which priming with lentogenic strain, followed by vaccination with experimentally produced killed vaccine having local velogenic NDV strain and followed by boosting with lentogenic strain showed maximum protection (92.85%) against the challenge of local velogenic NDV isolate.

Key words: Newcastle disease, Pathotype, Phylogenetic tree, Vaccine.

INTRODUCTION

Newcastle disease (ND) is one of the major concerns of poultry industry in the world. It is caused by Newcastle disease virus (NDV), a member of the *Paramyxoviridae*, genus Avulavirus and serogroup Avian Paramyxovirus 1 (Lamb *et al*., 2005). The virus is enveloped, and has negative-sense single-stranded genome of approximately 15 kb, in length which codes for six structural proteins, including Nucleocapsid Protein (NP), Phosphoprotein (P), Matrix protein (M), Fusion protein (F) and Hemagglutinin-Neuraminidase (HN) and large RNA-dependent polymerase protein (L) and two non-structural proteins W and V that are additionally created within the P gene during transcription of mRNA at editing site by insertion of guanines (Alexander, 2003). This important pathogen remains havoc for the poultry industry and causes a severe economic impact in terms of heavy production losses and high mortality. The disease effects 27 of the 50 orders of the birds (Madadger *et al*., 2013). Depending upon the pathotype and susceptibility the mortality varies from zero to 100% (Nanthakumar *et al*., 2000).

Isolates of NDV are categorized into three main pathotypes depending on the severity of disease produced by the isolate in chickens (Alexander 1989, 1991). Lentogenic isolates do not usually cause disease in adult birds and are considered avirulent. Viruses of intermediate virulence that cause respiratory disease are termed as mesogenic, while virulent viruses causing high mortality are termed as velogenic. Neurotropic and viscerotropic forms of velogenic viruses have been reported worldwide (Alexander, 1991).

New castle disease is endemic in Pakistan causing heavy economic losses to the National industry. Different ND outbreaks during 2011-2013 caused loss of worth approximately USD 200 million every year (Siddique *et al*., 2013). These viruses were isolated and typed as virulent on the basis of their Mean Death time (MDT), Intra Cerebreral Pathogenicity Index (ICPI) and cleavage motifs in fusion protein. Phylogenetic analysis of Fusion (F), Heamaglutnatinin Nuraminidase (HN) and Matrix (M) genes indicated the emergence of novel genetic group within linage-5. Several mutations were found in neutralizing epitopes of F and HN gene so there is a need for re-evaluation of currently used vaccines and vaccines practices (Munir *et al*., 2012).

It is widely recognized that an efficacious Newcastle disease (ND) vaccine made with any NDV does induce protection against morbidity and mortality from a virulent NDV challenge. However, the ND vaccines do not protect vaccinates from infection and viral shedding from such a challenge (Chukwudi *et al*., 2012). The anti NDV titers of the flocks were routinely monitored and revaccination was done to maintain the titers in satisfactory limits in case of any outbreaks. In breeder flocks vaccination is done prior to production, so that maternal antibodies prevent the progeny in first week of life. Inspite of all efforts ND is still prevailing in

poultry flocks (Shabbir *et al*., 2012). To overcome these problem different combinations of live and killed ND vaccines are used in the field.

Vaccines prepared from ND viruses corresponding to five different genotypes were compared to determine if the phylogenetic distance between vaccine and challenge strain influences the protection induced and the amount of challenge virus shed. The vaccine homologous with the challenge virus reduced oral shedding significantly more than the heterologous vaccines. NDV vaccines formulated to be phylogenetically closer to potential outbreak viruses may provide better ND control by reducing virus transmission from infected birds (Patti *et al*., 2007).

The present study was undertaken to analyze the biological nature of Newcastle disease virus (NDV) circulating in poultry population of Pakistan. Different Vaccines and vaccination schedules were also evaluated to know the possible way to control the disease.

MATERIALS AND METHODS

Samples were obtained from 22 poultry farms (13 broiler, 6 layer and 3 breeder flocks) showing sign and symptoms of Newcastle Disease. The flocks were from Rawalpindi, Islamabad, Attock, Chakwal, Mansehra, Abbatabad, Gujranwala, Lahore and Sargodha cities. The collected samples were trachea, spleen, lung tissues and cloacal& tracheal swabs (Haque*et al*., 2010). Tissue sample were homogenized in Phosphate Buffer Saline (PBS) and both the tissue and swab samples were filtered through syringe filters of 0.45 to 0.2 µm size. All of these samples were inoculated into the allantoic cavity of 9 to 11 day old chicken embryos and the allantoic fluids were harvested after 48 hrs. Samples were stored at -70^{o}C until further use (OIE, 2012)

Haemagglutination (HA) and Haemagglutination Inhibition (HI) assay: Allantoic fluids that were harvested from the embryos were tested for haemagglutination activity. For this purpose 25µl of fresh allantoic fluid was added in 1st well of microtiter plate and 2 fold serial dilutions were made in the PBS up to 11th well. 25µl of 1% washed Red Blood Cells (RBSs) were added up to 12th well and plates were incubated for 30 minutes at room temperature. Reciprocal of the last antigen dilution giving a clear lawn of evenly spread layer of RBCs were designated as antigen titer. 4 Haemagglutinating Unit (4 HAU) was calculated by dividing the end dilution point of testing antigen by 4. The samples showing haemagglutination activity were confirmed by haemagglutination inhibition assay. For this purpose 25µl of Newcastle Disease virus positive serum (VLA Scientific Cat. No. RAB0155) was serially diluted in PBS. 4 HAU of virus was added and plates were incubated. 25µl of 1% washed RBCs was added and the allantoic fluids showing haemaglutination inhibition by Newcastle disease virus positive serum was considered as positive (Alexander, 1989 and WHO, 2002).

Reverse transcriptase-Polymerase Chain Reaction (RT-PCR): Samples confirmed by HI test, were subjected to RT-PCR. RNA was extracted from allantoic fluids using the TRIzol reagent (Life Technologies Cat. No. 15596-026) following manufacturer's protocol. cDNA was synthesized by Reverse Transcription Kit (Finzymes, DyNamo^{TM} ,Cat. No. F-470L). NDV-F gene was amplified by using commercial Master Mix Kit (Finzymes, 2 x DyNAzyme^{TM}-II, Cat. No.508L).The primers used were forward (4306F) 5'GACCGCTGACCACGAGGTTA'3 and reverse (5005R) 5'AGTCGGAGGATGTTGGCAGC'3 (Aldous *et al*., 2003). The position 4554 to 4917 of NDV-F gene was targeted by these primers. The cycling parameters were 94°C for 5 minutes followed by 40 cycles of 94°C for 15 s, 56°C for 30 s, and 68°C for 1 min, final extension step was 68°C for 5 min. The PCR products were electrophoresed on 1.5% agarose gel and DNA bands were visualized under UV light after staining with eithidium bromide (Merino *et al.,* 2009).

Assessment of pathogenicity: Out of 22, 18 samples were confirmed as NDV. Four of the isolates named Chicken/ Broiler1/ Rawalpindi/ 2012, Chicken/ Broiler2/ Rawalpindi/ 2012, Chicken/ Broiler Breeder/ Rawalpindi/ 2012, Chicken/ Broiler /Attock /2012, that caused 38.33, 41.66, 43.75 and 58.33% mortalities were selected on the basis of the highest mortality for the assessment of pathogenicity. In vivo pathogenicity was measured by MDT in chicken embryos and ICPI in day old chicks.

Mean Death Time (MDT): The four selected isolates had HA titers more than 16. A 10 fold serial dilution of each of the four samples was made in PBS as 1/10, 1/100, 1/1000,1/100000000. All the dilutions were filtered sterilized without addition of antibiotics. The MDT was performed following standard protocols (Alexander, 2003; OIE, 2012). The diluted samples were inoculated into 10 days old chicken embryovia chorio-allantoic sac route. For each dilution 5 embryos were used and each of the 5 embryos was inoculated with 0.1 ml of the dilution. The embryos were incubated at 37^{o}C and candling was done at 6 hours intervals for 5 days and death of the embryos were recorded.

Intra cerebral pathogenicity index (ICPI):ICPI index test was performed in day old chicks. The ICPI test was performed following standard protocols (Alexander, 2003; OIE, 2012). The dilutions were injected intracerebrally to the chick. For each dilution 5 chicks were used and each of the 5 chicks was inoculated with 0.05 ml of the dilution. These chicks were examined after

every 24 hours for 8 days and every death and abnormality was recorded.

Preparation of experimental vaccine: The isolate chicken/broiler/Attock/2012 had the maximum pathogenicity index and the vaccine was prepared from it. The viral sample was 10 fold diluted in PBS and inoculated in 10 day old embryos. After 48 hrs the allantoic fluid was harvested and HA titer was calculated. LD_{50} was calculated by Reed and Munch method. Then the virus was killed by adding 0.2% formaline and the preparation was then diluted up to LD_{50} 10^8. Next it was diluted 1:1 with oil adjuvant Lipofundin®. Sterility test of the vaccine was performed on nutrient agar, sabourauds agar and freys broth and safety test was performed in chicken embryos by inoculating 0.1 ml of the preparation without oil adjuvant added (RAP, 2002).

Potency testing of the vaccine: Vaccine trials were carried out in day old broiler chicks splitted into 7 groups containing 42 birds each. All birds were kept in isolation and under strict biosecurity measures providing ad-libitum feed and water. Six groups were injected with the vaccine and one group was kept as unvaccinated control. All the six groups were vaccinated with different combinations of lentogenic (live La Sota), mesogenic (Mukteshwar) and velogenic (Chicken/ broiler/ Attock/ 2012) NDV strains. The routes of vaccination were eye droppings (E/D), in drinking water (D/W) or as injection 0.2 cc subcutaneous (S/C) in neck. These birds were vaccinated at days 1, 10 and 21 of age according to the strategy given in Table 2. The blood was collected from the birds on days 1, 9, 20 and 27 of age. Antibody titers were measured using the HI test. All the birds were challenged with the vaccine isolate (chicken/ broiler/ Attock/ 2012) at day 28 of age (Abbas *et al*., 2006). Birds were kept under observation for 10 days post challenge to record any clinical signs or abnormality.

RESULTS

Out of 22, samples analyzed 18 were confirmed to be ND through HA/HI test. These samples were further subjected to NDV specific RT-PCR of F gene. All the 18 samples showed bands of expected size of 700 bp PCR product confirming the presence of NDV (Fig 1). Four of these isolate i.e. Chicken/Broiler1/Rawalpindi/2012, Chicken/Broiler2/Rawalpindi/2012, Chicken/Broiler Breeder/Rawalpindi/2012 and Chicken/Broiler /Attock /2012, produced highest mortalities 38.33, 41.66, 43.75 and 58.33% respectively and ICPI was more than 1.5 showing they were velogenic NDV (Table 1).

The isolate of the maximum pathogenicity i.e the Chicken/broiler/Attock/2012 was used to prepare a formalized oil adjuvant vaccine. At the commercial poultry farms in Pakistan it is a practice that combination of live and killed ND vaccines are given to the birds in different schedules to protect them from the disease. Following the same convention we vaccinated the birds with lentogenic and mesogenic strains and our experimental vaccine. The group 1 of the birds was given live Lasota only, group 2 was given live Lasota and live Mukteswar, group 3 was given killed Mukteswar and live Lasota, group 4 was given killed chicken/ broiler/ Attock/ 2012 and live Lasota, group 5 was given live Lasota and Killed Mukteswar, group 6 was given live Lasota and Killed chicken/ broiler/ Attock/ 2012, as per strategy shown in Table 2. The group 7 contained unvaccinated controls. To measure the antibody titers the HI test was performed on sera and $\log_2$ of HI titers were calculated and sown in Table 3. Results of the challenge experiment as shown in Table 4 showed that the best protection, from challenge with the chicken/broiler/Attock/2012 strain given through intranasal route, was seen in group 6 (92.85 %). followed by followed by group 5 (85.7%), group 3 and group 4 (83.33%), group 1 and group 2 (78.57%) and unvaccinated control group 7 (2.38%) respectively.

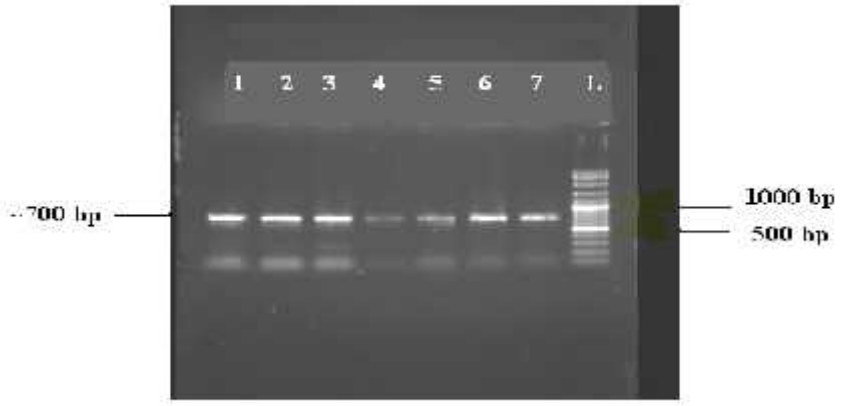

Figure1. PCR amplificatoin of F protein gene of NDVWell 1 to 7 represent 700bp PCR fragment of Fusion (F) protein gene and in well 8 represents 100bp ladder (Gene Ruler, Fermentas, USA)

Table 1. Complete History, HA titers and ICPI values of each NDV isolate.

Name of virus	Birds age	No. of birds	Location	Date of outbreak	HA titer Log_2	MDT	ICPI	Viral type
Chicken/Broiler1/Rawalpindi/ 2012	30 days	30000	Rawalpindi	15/05/20/2012	9	45.4	1.70	Velogenic
Chicken/Broiler2/Rawalpindi/ 2012	22 days	60000	Rawalpindi	20/05/2012	10	44.8	1.75	Velogenic
Chicken/Broiler Breeder/Rawalpindi/2012	42 days	8000	Rawalpindi	28/03/2012	9	46.2	1.72	Velogenic
Chicken/Broiler /Attock /2012	30 days	30000	Attock	11/01/2012	10	44.6	1.75	Velogenic

Table 2. Vaccination Strategy in experimental groups.

Group No.	Day of vaccination			
	Day 1	**Day 10**	**Day 21**	**Day 28**
1	Lasota (E/D)	La Sota (E/D)	La Sota (D/W)	Velogenic field
2	Lasota (E/D)	Mukteswer (E/D)	La Sota (D/W)	strain
3	Mukteswer Killed injection + Lasota (E/D)	La Sota (E/D)	La Sota (D/W)	challenge
4	Velogenic Killed injection + Lasota (E/D)	La Sota (E/D)	La Sota (D/W)	
5	Lasota (E/D)	Mukteswer Killed injection + La Sota (E/D)	La Sota (D/W)	
6	Lasota (E/D)	Velogenic Killed injection + La Sota (E/D)	La Sota (D/W)	
7 (Control)	No vaccine	No vaccine	No vaccine	

Table 3. Mean log2Haemagglutination Inhibition antibody titers of birds before and after Vaccination.

Group No.	Age in days			
	Day 1	**Day 9**	**Day 20**	**Day 27**
1	6	3.2	3.8	4.8
2	5.2	3.8	4.2	5.0
3	6.8	3.2	3.4	5.4
4	5.4	2.4	3.6	5.6
5	6.2	4.2	4.4	5.2
6	6.4	3.6	3.2	5.4
7	6.2	3.2	2.8	1.8

Table 4. Mortality in chicken after challenge with velogenic strain (chicken/broiler/Attock/2012).

Group No. n=42	Mortality within 10 days	Total birds survived	% protection
1	10	33	78.57
2	9	33	78.57
3	8	35	83.33
4	7	35	83.33
5	7	36	85.7
6	3	39	92.85
7	41	01	2.38

Protection% rates were indicated by numbers of birds survived/numbers of birds challenged X 100.

DISSUSION

ND has been causing huge economic losses in Pakistan since 2010. Most of the outbreaks occurred in commercial broiler, breeder and layer flocks that were affected at rearing age. Some outbreaks were also reported in rural poultry and in peacocks. Rate of mortality was variable ranging from 0 to 100%. Previously the magnitude of the wave of NDV outbreaks was small and the disease was limited to some areas of Punjab only. But from November 2011 onwards the disease spread throughout the country and caused huge mortality in commercial as well as rural poultry. In a previous study eight Pakistani NDV isolates were characterized (Munir *et al*., 2012). Several new mutations were observed in the F and HN genes and phylogenetic analysis indicated that these viruses belonged to a new group within lineage 5. These findings pointed to the need for re-evaluation of the vaccines and vaccination programs currently in use. The results also emphasized the importance of continuous surveillance of the disease.

This study was designed in view of the current epidemic of ND. The study is focused on isolating the virus from field condition and characterizes them at biological level and to suggest the vaccine regime that gives better protection during epidemics. We analyzed 22 samples selected from the suspected cases, out of which 18 were confirmed as ND based on HA/HI tests and PCR. Pathogenicity testing by the MDT and ICPI tests showed four of the isolates were of velogenicpathotype. ICPI is the most reliable and reproducible test providing a good indication of relative virulence of the viruses (Hanson,1980 and Cattoli*et al*., 2011). During the current epidemic of ND in Pakistan different vaccination schedules have been employed at the commercial poultry farms. Combination of live and killed vaccine may be a solution to control the disease (Folitse*et al.* 1998). We prepared a killed oil adjuvant vaccine from the most virulent isolate (chicken/broiler/Attock/2012) from amongst the strains isolated in this study. Keeping in view the practice in at the commercial farms, we inoculated our experimental birds with different schedules that combined commercial live and killed vaccines and our experimental vaccine. Analysis of variance was used to show that anon-significant difference existed in the HI titers induced by the different combination of vaccines, those birds that were vaccinated with combinations of live and killed vaccines gave better protection. The schedule 6, in which Lasota live (E/D) at day 1, chicken/broiler/Attock/2012 killed (injection) + Lasota live (E/D) at day 10 and Lasota live at day 21, gave the best protection. This was followed by the schedules 5 and 4. Schedule 5: Lasota live (E/D) at day 1, Mukteswer killed (injection) + Lasota live (E/D) at day 10, Lasota live (D/W) at day 21, schedule 4:chicken/broiler/Attock/2012 killed (injection) + La Sota live (E/D) at day 1, Lasota live (E/D) at day 10, Lasota live (D/W) at day 21. Next was schedule 3: Mukteswer killed (injection) + Lasota live (E/D) at day 1, La Sota live (E/D) at day 10, La Sota live (D/W) at day 21 respectively. Minimum protection was seen in group 1 and 2 that had been inoculated with live lentogenic (Lasota) or mesogenic (Mukteswer) vaccines. These results indicate that a combination of vaccines in which priming is done with a live attenuated vaccine at day 1, followed by boosters with a killed vaccine at day 10, and with a live vaccine at day 21 give the best protection. Rahman *et al*., 2002 suggests that vaccination against ND with live vaccines should be done at 8 to 10 days of age to prevent neutralization of maternal antibodies. Some researchers are of the view that vaccination should be practiced at day first to produce active immunity (Voeten*et al*., 1987). It revealed that the vaccine prepared from the local strain of virus may better protect the birds during field outbreaks. Moreover it also reduces the virus shedding and hence will help to control the disease (Patti *et al*., 2007).

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