Efficacy of experimentally prepared oil-based Newcastle disease (ND) vaccine (Mukteswar strain) against prevailing virulent ND virus in Punjab, Pakistan

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Abstract:
Oil-based inactivated Newcastle disease (ND) vaccine was prepared and its efficacy against the prevailing velogenic ND virus was determined. Oil-based vaccine was prepared by mixing one part of the inactivated antigen with three parts of the montanide oil. The vaccine was evaluated for its safety, stability and immunogenicity. One hundred and twenty five day old birds were divided in 5 groups designated as A to E. The birds of different groups were treated with experimentally prepared vaccine alone and in combination with live ND vaccine (mukteswar) at different age by using different dose rate and routes of administration. The anti-NDV-HI antibody response of all the four groups was determined on day 14, 21, 28, 35 and 42 post-vaccination. On 28th day post vaccination, the birds were challenged with velogenic field isolated virus. The birds that survived from challenge were also bled at day 42 of age to determine vaccine response. High antibody titers and 100% protection was observed in birds of group B which suggested that simultaneous use of both live and killed oil-based vaccines at day 7th of age is helpful in the prevention against disease challenge. In A, C and D groups 90% protection was seen. Oil-based ND vaccine containing Mukteswar strain gave remarkable antibody titers to resist the field virus. So it was concluded that oil based vaccine can give better immune response and protection against disease when used in early age in broiler chicks.

Key Words: Newcastle disease, Mukteswar, oil based vaccine, Paramyxovirus, challenge.

Introduction

Newcastle disease (ND) is a highly fatal and contagious viral disease of birds which affects wild, aviary avian species and the domestic poultry. ND emerged both in endemic and epidemic form all over the world (Brown et al., 1999). It has been observed that about 250 species of the birds are affected with this disease irrelevant of their age groups. As chicken are most susceptible to ND, great economic losses are contributed by ND in poultry industry (Alexander et al., 2003). ND has been ranked among most infectious diseases of great importance by the world organization for animal health (Singh et al., 2005; Linde et al., 2010). First outbreak of ND was reported in Indonesia (Kranveld et al., 1926) and then in Newcastle-upon-Tyne, town of England (Doyle et al., 1927). The disease was reported at Ranikhet in India from where it got its name “Ranikhet”. In Pakistan the disease remained as an endemic since it was reported for the first time in Southeast Asia in 1926. It was reported in many poultry producing areas of Pakistan throughout the year in sporadic form (Muneer, 1996). ND virus in combination with avian influenza virus strains like H5, H7 and H9 is producing disastrous effects on the poultry industry in Pakistan (Naeem et al., 2003). The etiology of ND is Avian paramyxovirus -1 (APMV-1) also called as Newcastle disease virus (NDV) which belongs to genus Avulavirus and family Paramyxoviridae (Mayo et al., 2002). NDV genome is negative sense, single stranded RNA molecule containing 15,186 nucleotides. Complete genome sequencing and a phylogenetic study of a NDV isolate in Pakistan revealed that it belongs to Genotype VII and more closely to sub genotype VIIb and was named as a new genotype called VIIf (Munir et al., 2012).
The signs and symptoms of ND include greenish diarrhea, coughing, sneezing, rales and gasping. Twisting of the neck, circling, drop in egg production, dropping wings, legs paralysis, torticollis and swollen tissues around neck and eyes are the other signs observed in this disease (Wakamatsu et al., 2006).

In Pakistan both live and inactivated vaccines are being used to control the disease. There are many vaccines available including locally prepared and imported with different strains e.g. La Sota, F and Mukteswar etc. (Rehmani, 1996). The use of imported live vaccines may pose a threat to the poultry industry as mutations may result in the emergence of highly virulent virus from less virulent one. This might be the case of the recent outbreaks in Pakistan. Furthermore, there is no standard vaccination schedule available, generating a need of legislation along with strict monitoring. Failure of imported vaccines having La Sota strain to protect the birds against the field infection particularly in broilers is of great concern. Inactivated NDV vaccines having mineral oil have been used frequently in Pakistan since 1973 (Stone et al., 1978; Cajavec et al., 1996). Adverse effects associated with inactivated vaccines have been greatly reduced using aqueous and oil-phase emulsifiers that lead to enhanced stability and persisting immune response of the vaccine (Stone et al., 1983; Stone, 1988). In spite of massive vaccination ND outbreaks during the 2011 and 2012 affected almost 45 million of poultry among which almost 44 million poultry died away. The acute disease outbreaks have been observed on commercial poultry farms affecting all types of birds. In commercial poultry 75% incidence of the disease is seen after 20th day of age and 25% incidence before 20th day of age (Anonymous, 2012). This critical condition necessitated the production and evaluation of inactivated oil-based ND vaccine (Mukteswar strain) against the prevailing velogenic NDV isolated from current outbreaks.

**Materials and Methods**

**Vaccine Preparation**

Newcastle disease virus (Mukteswar strain) to be used for vaccine production was taken from working seed bank of Poultry vaccine section of Veterinary Research Institute (VRI) Zarzar shaheed road Lahore, originally preserved in freeze dried form. The freeze dried seed virus (0.5ml pellet) was reconstituted in 1ml PBS having pH 7.2-7.4 and given 3 passages in 9 days chicken embryonated eggs. For vaccine preparation 0.1ml of 10^-4 dilution of the allanto-amniotic fluid (AAF) collected from passage 3 was inoculated into 09 days chicken embryonated eggs according to the method explained by Senne (1989). The virus infected eggs were incubated at 37°C and all the eggs with dead embryos within 44-60 hours after inoculation were chilled overnight. The AAF was collected from dead embryos according to the method explained by Hitchner et al., 1980 with few modifications. Safety test of the AAF was performed by culturing onto nutrient agar, macConkey agar, thioglycolate agar and in mycoplasma broth. The cultured media were kept at 37°C. The mycoplasma broth was observed for 10 days for any growth etc. The haemagglutination (HA) test of the AAF was also performed following the procedure described by Maff, 1984.

The virus neutralization test of the AAF was also performed for the confirmation of ND virus using the reference anti-NDV-sera procured from OIE reference laboratory Pirbright, UK. The collected AAF was pooled in sterilized glass flasks. Formalin (37%) was added by 0.1% v/v. fluid. The fluid was mixed thoroughly and incubated at 37°C for 24 hours. Penicillin 10 Lac 100ul/ml, streptomycin 1mg/ml, Gentamycin (200mg/ml) and Nystatin (1000 units per ml) were added to prevent the bacterial and fungal growth. Then it was stored at -4°C in the refrigerator for further use. After inactivation HA and sterility tests were performed as described above. The inactivated material was subjected to safety test by injecting 0.2ml of the inactivated AAF into 9 days embryonated eggs through allantoic cavity.

Oil-based vaccine was prepared by mixing two parts of the inactivated AAF into 4 parts of the montanide oiladjuvant. The mixture was homogenized at 3000 rpm for 15 minutes for proper mixing. The prepared vaccine was dispensed into the plastic bottles and was stored at +4°C.

The prepared vaccine was checked for its stability by storing at +4°C for three months. Safety of the vaccine was tested by injecting 2ml of the vaccine into five chicks. The birds were kept under observation for 15 days for any signs and symptoms of the disease. The sterility of the vaccine was checked by culturing the vaccine onto different media.
Vaccine Efficacy testing
A total of 125 day old broiler chicks were procured from market and kept under standard conditions of sanitation in experimental units of poultry vaccine section of VRI. The birds were offered feed and water ad libitum and divided into 5 groups randomly mentioned as A, B, C, D and E having 25 birds each.
Sixteen birds from each group were bled randomly at day zero to collect sera for maternal antibody detection. Sera were stored at -20°C after treating it at 56°C for 30 minutes for the inactivation of nonspecific proteins and processed for anti-NDV-antibody titer calculation.
Birds of group A were vaccinated with experimentally prepared vaccine of ND @ 0.3ml per bird by sub-cutaneous injection at the neck region and ND- live (Mukteswar) intraocularly. At day 7th birds of group B were vaccinated using 0.3ml of experimentally prepared ND vaccine per bird intramuscularly and with live ND (Mukteswar) vaccine through intra-ocular route simultaneously. Birds of group C were vaccinated with experimentally prepared vaccine at day zero of age by sub-cutaneous route and at day 7th with live ND vaccine via intraocular route. In group D the birds were injected with experimentally prepared vaccine by injecting 0.3ml per bird through sub-cutaneous route at day 7th of age. While the birds of group E were kept as unvaccinated control (Table-1).
Blood samples were collected from 16 randomly selected birds of each group at day 14, 21, 28, 35 and 42 of age. The serum samples thus collected were subjected to haemagglutination inhibition (HI) test following the procedure described by MAFF, 1984 to find anti-NDV antibody titers.
ND Virus isolated from current ND outbreaks in Punjab during 2012 was used in challenge study. The biological pathotyping of the virus confirmed the virulent nature of the virus. The mean death time and intra-cerebral pathogenicity index of the virus was 49.6 hours and 1.65 respectively. Titration of the virus was done to determine the embryo lethal dose 50 (ELD50) following the method of Reed and Munch, 1938.
Seven birds of each group were infected with challenge virus dose of 100 x ELD50 at day 28 of age through intraocular route. For this purpose virus was inoculated in 9 days embryonated eggs &1 ml of the freshly harvested fluid was diluted in 99 ml of normal saline. Again 1ml from first dilution was mixed with 99ml sterile normal saline to make 10^-3 dilution. A dose of 100 x ELD50 of 10^-4 dilution was used for challenge in each bird. The birds challenged with field virus were kept under observation for any sign and symptoms of the disease for 15 days. Any mortality and morbidity was recorded. Postmortem of dead birds conducted to record the lesions. Healthy vaccinated birds were slaughtered to see effect of challenge virus on body of the birds. The challenged birds that survived were bled to collect serum for anti-NDV-antibody titer determination on 35 and 42 day of age. Post challenge HI titer was determined to correlate the post challenge antibody titers with the protection and mortality. GMT calculation was done following the procedure described by Villegas and purchase (1989).

Table 1. Experimental design

<table>
<thead>
<tr>
<th>Group</th>
<th>Age (days)</th>
<th>Vaccine</th>
<th>Dose/Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>Experimentally prepared ND vaccine</td>
<td>0.3ml sub-cutaneous</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ND-live vaccine (Mukteswar strain)</td>
<td>Intraocular</td>
</tr>
<tr>
<td>B</td>
<td>7</td>
<td>Experimentally prepared ND vaccine</td>
<td>0.3ml Intramuscular</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ND-Live vaccine(Mukteswar strain)</td>
<td>Intraocular</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>Experimentally prepared ND vaccine</td>
<td>0.3ml sub-cutaneous</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>ND-live vaccine (Mukteswar strain)</td>
<td>Intraocular</td>
</tr>
<tr>
<td>D</td>
<td>7</td>
<td>Experimentally prepared ND vaccine</td>
<td>0.3ml sub-cutaneous</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td>Control</td>
<td></td>
</tr>
</tbody>
</table>

Results and Discussion
The AAF harvested for vaccine preparation was found sterile as no growth was seen on cultured media. The HA titer and ELD50 were found to be 512 and 10^-8.16 respectively (Table-2). As the inactivated vaccines are produced by
inactivating the virus with B-propiolactone or formalin. In this study the NDV suspension was inactivated using formaldehyde (Merck: 37%) at rate of 0.1%. The HA titer of the inactivated material was decreased by one log from 512 to 256 and the inoculated embryos remained alive. The inactivated virus did not killed 9 days embryos even after four passages. The AAF collected as optically from live embryonating eggs of fourth passage did not gave HA activity. Formalin has the activity of inactivating proteins and nucleic acids by acting on amino, amide groups and on the non-hydrogen bonded amino groups in purine and pyrimidine bases respectively. In this way cross links are made that confer structural rigidity (Jagt et al., 2010).

Table 2: Calculation of ELD_{50} of working Seed virus:

<table>
<thead>
<tr>
<th>Group No.</th>
<th>AAF Dilution</th>
<th>Total No. of eggs.</th>
<th>NO. of dead embryos</th>
<th>NO. of live embryos</th>
<th>Cumulative Dead</th>
<th>Cumulative Live</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>$10^{-5}$</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>13</td>
<td>1</td>
<td>13/14=92.85</td>
</tr>
<tr>
<td>B</td>
<td>$10^{-6}$</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>9</td>
<td>2</td>
<td>9/11=81.81</td>
</tr>
<tr>
<td>C</td>
<td>$10^{-7}$</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>5/9=55.55</td>
</tr>
<tr>
<td>D</td>
<td>$10^{-8}$</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>7</td>
<td>2/9=22.22</td>
</tr>
<tr>
<td>E</td>
<td>$10^{-9}$</td>
<td>(5)</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

$10^{-7}$ dilution gives mortality immediately above 50% that is 55.55%
$10^{-8}$ dilution gives mortality immediately below 50% that is 22.22%

Index = \frac{\text{Percentage infected immediately above 50%} - 50}{\text{Percentage infected above 50%} - \text{Percentage infected below 50%}}

Index = \frac{55.55 - 50}{55.55 - 22.22} = \frac{5.55}{33.33} = 0.16

ELD_{50} = \text{Negative log of dilution above 50%} + \text{Index}

ELD_{50} = 7 + 0.16 = 7.16

This is the titer of virus per 0.1 ml of the AAF inoculated per egg.

ELD_{50}/0.1ml = 7.16

ELD_{50}/ml = 8.16

In this study the NDV suspension was inactivated using formaldehyde (Merck: 37%) at rate of 0.1%. These findings of inactivation are in accordance with the work of Nisar et al. (2011) in which they studied the inactivation of avian influenza virus using formaldehyde, Razmaraii et al. (2012) in which formaldehyde was used for the inactivation of ND virus for vaccine production and Mudasser et al. (2006) who used formaldehyde for the inactivation of Infectious Bursal Disease (IBD) virus in their study.

Inactivated viral suspensions when are absorbed rapidly from the injection site without causing the stimulation of the immune-competent cells. Such antigens are not taken and processed by the antigen presenting cells, so proper development of memory or plasma cells does not take place (Abbas et al., 1991). To enhance the immunogenicity of such vaccines, adjuvants are added in inactivated NDV suspensions as they increase the efficacy of vaccines by rendering the antigens more purified. Effective inactivated vaccines are produced by the addition of an oily formulation (Dupuis et al., 2006). There are different types of adjuvants each having different way of enhancing the immunogenicity of the antigen (Aucouturier et al., 2001). Therefore montanide ISA 70 oil was used in this study as
adjuvant to the prepare water in oil in water (w/o/w) emulsion. This adjuvant was selected to be used to prevent the side effects like granuloma formation etc. at the site of injection associated with the use of other mineral oil emulsions (Hafeez et al., 2011). In this study Montanide oil (Montanide ISA 70) was used as adjuvant that gave good immune response. No inflammatory reaction was observed at the site of injection. In their studies (Ayesha et al., 2005; Iqbal et al., 2008; Aslam et al., 2012) also used Montanide oil ISA 70 in the preparation of oil-based vaccines and got better results both in terms of immunity and prevention of side effects.

A homogenous suspension was obtained by mixing two parts of the inactivated AAF with 4 parts of the montanide oil. The safety of the prepared vaccine was found clear as all the chicks injected with increased dose of the vaccine remained alive without showing any clinical sign and symptoms. The vaccine remained stable at +4°C without the separation of oil and antigen phase. The ELD50 of the virus used in challenge study was 10⁻⁶.⁵/ml.

The Geometric mean titer (GMT) of anti-NDV-antibodies of the birds at day zero of age was 222.4. The GMT of anti-NDV-antibodies at day 14, 21, 28, 35 and 42 of age of the birds of group A were 4.3, 36.8, 104.0, 32.0 and 22.6, of birds of group B were 4.3, 36.8, 104.0, 32.0 and 22.6 of birds of group C were 5.7, 22.6, 42.2, 36.8 and 11.3 and for group D were 6.1, 16.0, 18.4, 11.3 and 8.0 respectively (Table-3 and Figure-1). These results show that maximum HI titters of the birds were shown when both the live and inactivated oil-based ND vaccines were given simultaneously at day 7th of age. Simultaneous vaccination gave good results as oil-emulsion vaccine response is slowed down due to the granuloma formation and in this period live vaccine primes the birds. Then boosting is by the slow release of the antigen from the granuloma of oil-based inactivated vaccine. Comparing with other programs, this vaccination schedule gave high GMT value of 36.8 and 104 at 21 and 28 days of age respectively. This result indicates that vaccination at the early age with both live and killed vaccines in combination can protect the broilers against the disease by evoking sufficient immune response. In this way, the labor cost is saved and handling stress on the birds is also minimized. These findings are in line with the findings of other workers (Edison et al., 1982; Foltise et al., 1998).

**Table 3.** Comparison of Geometric Mean HI antibody titers of all groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days with GMT values</th>
<th>Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>A</td>
<td>222.4</td>
<td>11.3</td>
</tr>
<tr>
<td>B</td>
<td>222.4</td>
<td>4.3</td>
</tr>
<tr>
<td>C</td>
<td>222.4</td>
<td>5.7</td>
</tr>
<tr>
<td>D</td>
<td>222.4</td>
<td>6.1</td>
</tr>
<tr>
<td>E</td>
<td>222.4</td>
<td>3.5</td>
</tr>
</tbody>
</table>
The vaccines efficacy is tested mainly by determining the level of antibodies produced in the vaccinated birds and their ability to resist the challenge of virulent virus when compared with unvaccinated birds (Allan et al., 1978; Spradbrow, 1994). In this study, the vaccinated birds were challenged with the virulent virus isolated and biological characterized from recent ND outbreaks at 28 days of age. Among challenged birds 90% protection was seen in birds of groups A, C and D while 100% protection was seen in birds of group B. It means that the inactivated vaccine alone and in combination with live vaccine provoked better immune response and protected the birds from disease. In these groups HI titer was up to 32 or above. These titers proved to be protective against challenge infection. In control group where 100 % mortality occurred, HI titers were very low. Few studies (Schmidt et al., 1995; khan et al., 2011; Bawla et al., 2011) also reported that HI titer 32 or above gives protection against disease. Post challenge antibody titers of the survived birds at day 42 of age were 445.7, 558.1, 274 and 388.0 for group A, B, C and D respectively. These high antibody titers in protected birds were due to the challenge virus.

In control group, the birds showed decrease in MDA with the passage of time and it was 1.0 by the end of the 3\textsuperscript{rd} week of age. All the birds died away in this group within few days post challenge. These birds showed clinical signs and lesions that confirmed the Velogenic ND infection. In their studies (McCraken, 1988; Parede and young, 1990; Hamid et al., 1991; Bawla et al., 2011) also observed similar findings in their studies.

\textbf{Conclusion}

It was concluded that simultaneous use of both live and experimentally prepared inactivated oil-based vaccines (Mukteswar strain) at early age gives 100% protection against challenge infection with the prevailing virulent velogenic virus without provoking any adverse effect. This vaccination schedule gives persisting antibody titer to protect commercial broiler birds throughout their 42 days of age. This vaccination program is of great value in Pakistan because it supports both cell mediated and humoral immunity particularly in areas where ND outbreaks are high and the disease is in endemic form.
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