

## Development of Humoral Immune Response to Thermostable Newcastle Disease Vaccine Strain I-2 in Ring-Necked Pheasant (*Phasianus colchicus*)

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Article ID: KVFD-2020-25021 Received: 27.09.2020 Accepted: 11.01.2021 Published Online: 12.01.2021

### Abstract

Newcastle Disease (ND) was ranked as a List A and trade limited disease by the World Health Organization. It is one of the deadly diseases of domestic and zoo birds especially pheasants that are highly susceptible species to Newcastle disease virus (NDV). This study was designed to determine the humoral immune response (HIR) of thermostable NDV vaccine strain I-2 in same age pheasants. For this purpose, forty-five pheasants of the same age were separated and placed in cages. Thermostable NDV I-2 vaccine was mixed with feed and administered through oral route to the same age pheasants. HIR was detected using haemagglutination inhibition test (HI) and enzyme linked immunosorbent assay (ELISA) on 0, 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>th</sup> and 28<sup>th</sup> days post-vaccination (DPV). Optimum geometric mean anti-NDV-ELISA (2380) and anti-NDV-HI (Log<sub>27.5</sub>) antibodies titers were identified on 14<sup>th</sup> DPV. It was concluded that the oral administration of NDV I-2 strain is able to elicit a protective immune response in pheasants. Moreover, the use of this novel vaccine technique at the same age of pheasants overwhelms the attempt to catch these birds for single vaccination.

**Keywords:** Thermostable, Pheasant, Newcastle, ELISA, Vaccine

## Halka Boyunlu Sülün'de (*Phasianus colchicus*) Termotabil Newcastle Hastalığı Aşı Suşu I-2'ye Karşı Humoral İmmün Yanıtın Gelişimi

### Öz

Newcastle Hastalığı, Dünya Sağlık Örgütü'nün A listesinde yer alan ve ticareti sınırlayan bir hastalıktır. Evcil ve hayvanat bahçesi kuşlarının, özellikle de Newcastle Hastalığı Virüsüne (NDV) oldukça duyarlı türler olan sülünlerin ölümcül hastalıklarından birisidir. Bu çalışma, aynı yaşta sülünlerde termotabil NDV aşı suşu I-2'ye karşı şekillenen humoral immün yanıtı belirlemek için tasarlanmıştır. Bu amaç doğrultusunda kırk beş sülün ayrı ayrı kafeslere yerleştirildi. Yemle karıştırılan termotabil NDV I-2 aşısı, sülünlere oral yolla verildi. Humoral immün yanıt, aşılamadan sonraki 0, 7., 14., 21. ve 28. günlerde (DPV) Hemagglütinasyon İnhibisyon (HI) testi ve Enzim İşaretli İmmünosorbent Testi (ELISA) kullanılarak tespit edildi. Optimum geometrik ortalama anti-NDV-ELISA (2380) ve anti-NDV-HI (Log<sub>27.5</sub>) antikor titreleri aşı uygulamasını takiben 14. günde saptandı. NDV I-2 suşunun oral yolla uygulamasının sülünlerde koruyucu bir immün yanıt oluşturabileceği kanaatine varıldı. Ayrıca, aynı yaşta sülünlerde kullanılan bu yeni aşı uygulama tekniği, aşılama sırasında bu kuşların tek tek yakalanma girişimlerinin üstesinden de gelmektedir.

**Anahtar sözcükler:** Termotabil suş, Sülün, Newcastle Hastalığı, ELISA, Aşı

### How to cite this article?

Siddique F, Abbas RZ, Iqbal A, Rabbani M, Rafique A, Hussain I, Ahmed R, Mahmood MS, Lotfi A: Development of humoral immune response to thermostable Newcastle disease vaccine strain I-2 in ring-necked pheasant (*Phasianus colchicus*). *Kafkas Univ Vet Fak Derg*, 2021 (Article in Press). DOI: 10.9775/kvfd.2020.25021

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## INTRODUCTION

Small population of ring-necked pheasant (*Phasianus colchicus*) is bred as a game bird in Asia. They are primarily bred in various parts of Europe, America as a game bird. It belongs to family *Phasianidae* genus order *Galliformes* and subfamily *Phasianinae* [1]. Males are dominant, long tails and extremely decorated with bright colors as compared to females. Ring-necked pheasant may serve as biological indicator for the study of wildlife species and ecosystems fitness [2]. Fifty two species are present worldwide and six of these species are found in Pakistan. Most of the species are known to be endangered due to hunting and susceptibility to various diseases such as Newcastle disease (ND). Hunters choose this bird because of its higher quality of meat (high essential amino acid, fatty acids and low fat profile) as compared to ducks, broilers and geese [3-6]. Newcastle disease has been identified as one of the most pathogenic diseases of birds worldwide. [7,8]. This disease affects approximately two hundred fifty species of birds and results in high morbidity and mortality that may pose significant economic losses and limit trade and embargoes [9-11]. Ring-necked pheasants are more susceptible to ND virus and the virus often causes death [12]. The main causative agent of ND in pheasants is avian paramyxovirus serotype 1 (APMV-1), which belongs to the Paramyxoviridae family and the Avula virus genus [13]. It is a negative sense, enveloped, non-segmented and filamentous RNA virus with a genome size of 15.2 kb [14]. Contamination of feed and water with NDV containing bird's droppings is the main reason for the spread of this disease to healthy pheasant [15]. Flying birds, such as doves and pigeons, are also capable of spreading this virus to nearby pheasants and are primarily responsible for many recent epidemics [16,17]. Pheasants of all ages are susceptible to NDV and the average incubation period of NDV is 5-6 days [18]. The clinical signs and symptoms of pheasants infected with ND depend on many factors, including age, host immune status, virus strain and environmental conditions [19]. Many clinical symptoms of pheasants have been observed, such as white green diarrhea, head shaking, lack of appetite, reduced egg production, sneezing, coughing and trouble in breathing. In pheasants, mortality rate can easily reach to 100% since pheasants are known to have higher morbidity and mortality during the ND epidemic [12,20]. The control of Newcastle disease in pheasant reduced the risk of spread to commercial poultry [21]. In addition to biosecurity steps, vaccination is the most effective method to control the disease. There are two important concerns related to the vaccination of flying birds. First, if the cold chain system is not adequately managed when delivered to end users in warehouses and drug stores, the consistency of commercially available NDV vaccines would typically degrade rapidly. Secondly, administration of vaccines is very difficult in wild birds [22]. To counter these issues, Thermostable NDV vaccine has been prepared, and can be administered by mixing with feed, so there is no need to capture birds for

vaccination. Thermostable vaccine is a promising approach to control ND in pheasants as this vaccine maintains its efficacy at 28°C for 6-8 weeks and at 4-8°C for 1 year.

## MATERIAL AND METHODS

### Ethical Statement

The experiment was conducted under the regulations stipulated by the Independent Ethics Committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan (Number of bio-ethical committee letter is ORIC 1749, 1.6.2020).

### Study Area

The experiment was performed at Safari Zoo Lahore. The Park was established in 1982 and spreads over 242 acres. The park contains the country's largest walkthrough aviary.

### Source of NDV Vaccine Strain

The thermostable NDV I-2 strain was obtained from the Department of Microbiology, University of Veterinary and Animal Sciences, Lahore.

### Preparation of Vaccines

#### - In Ovo Propagation of Thermostable NDV

Five hundred (nine-day old) chicken embryos were procured from well reputed hatchery and transferred to an incubation facility available at Vaccinology Laboratory of the Department. Candling of embryos were done for evaluating livability of the embryos followed by proper labeling and documentation. After thorough scrubbing of egg shells using 70% ethanol, air sac was marked using lead pencil. A bore was made in few centimeters above air sac of egg shell of each embryo for inoculation of 0.1-0.2 mL of thermostable NDV strain (0.1 mL) under sterile environment. The bore was sealed using molten wax and re-incubated at 37°C. Candling process was repeated at 10<sup>th</sup> day after incubation and dead embryos were discarded. While on day 11, remaining embryos were transferred in refrigerator at 4°C for 6 h. The amniotic-allantoic fluid was collected and stored at -20°C for further processing [22].

#### - Titration of I-2 NDV Virus

Titration of I-2 NDV was calculated by Egg infective dose 50 (EID<sub>50</sub>). Briefly, tenfold serial dilution of virus was made from 10<sup>2</sup>-10<sup>10</sup>. The virus (0.1 mL) from each dilution was injected into nine days old chicken embryonated eggs. These eggs were incubated at 37°C for 24-72 h. The eggs were chilled at 4°C overnight. Allantoic fluid was collected from each egg and haemagglutination test was performed to examine the presence of the virus. The live thermostable ND virus was subjected to lyophilization for further use as oral vaccine/mixing with feeds.

**- Sterility Test**

Vaccine sterility was confirmed by culturing it on bacterial and fungal media for the presence of any contamination. For this purpose, 3 mL of vaccine fluid was centrifuged (500 xg) for 10 min and the residue was streaked on nutrient agar, MacConkey's agar, blood agar, and Sabouraud's dextrose agar plates. Loop full sediment was also inoculated in Frey's modified medium for Mycoplasma. These plates were incubated at 37°C for 48 h except Sabouraud's agar and Frey's medium. The Sabouraud's agar plates were incubated at 25°C in a humid chamber and Frey's medium plates were incubated for 7-10 days at a 37°C. All the plates were observed for microbial contamination<sup>[23]</sup>.

**- Safety Testing**

After the vaccine was proved to be sterile, each batch of vaccine was inoculated in 4-5 day-old 20 chickens through drinking water. The inoculated chickens were monitored for any pyrogenic effect, vaccine shock and/or vaccinal reaction.

**- Experimental Design**

Forty-five ring-necked pheasants of same age were separated from other birds at Safari Zoo Lahore. The birds were examined for antibodies against NDV and those which were found to be seronegative were included in the study. These birds were kept under standard management conditions. The forty-five pheasants were separated into three groups (G1, G2 and G3), fifteen pheasants in each. 5 mL of fresh, sterilized and non-chlorinated water has been added to the lyophilized NDV I-2 vaccine bottle (UVAS-CASTLE VAC) containing 10<sup>6</sup> Egg Infective Dose 50 per chicken. This viral suspension was further diluted in 50 mL water. The vaccine suspension was sprayed on feed (15 mL per 150 g of feed) and mixed thoroughly. Then, this vaccine treated feed (150 g) was spread on a clean cloth and fifteen pheasants from G1 were allowed to eat until the feed finished. Therefore, each pheasant ate approximately 10 g of vaccine coated feed. G2 was vaccinated with commercially available thermolabile NDV LaSota vaccine (Intervac Pvt. Ltd) as a positive control. The G3 was kept as a negative control (phosphate buffer saline) in the complete experimental design. Pheasants of each group were caged separately. The pheasants were checked daily two times (morning and evening) throughout the whole experiment to observe for any abnormal behavior<sup>[23,24]</sup>.

**- Collection of Samples**

Blood samples (2-3 mL) from each group were collected directly from brachial vein in sterile blood collection tubes. Samples were transported to Quality Operations Laboratory, University of Veterinary and Animal Sciences, Lahore and stored at -80°C for further use in serological tests.

**- Serological Test to Determine Humoral Immune Response**

The HIR in terms of anti-NDV antibodies were determined through Haemagglutination inhibition (HI) test and Enzyme-linked immunosorbent assay (NDV ELISA Kit, IDEXX Laboratories, Westbrook, ME) on days 0, 7, 14, 21 and 28 post-vaccination (DPV).

**Statistical Analysis**

The data for HI and ELISA tests were analyzed by calculating geometric mean titers (GMTs), mean±SD and through one-way analysis of variance (ANOVA) followed by Tukey's test using SPSS software (version 20.0).

**RESULTS**

In the present study, single feed mixing NDV I-2 vaccine developed protective antibody response in same age pheasants after seven days of post vaccination. Two main serological tests, HI and ELISA, were used to evaluate antibody titers. The outcomes of anti-NDV-ELISA antibody titers at 0, 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>th</sup>, and 28<sup>th</sup> days of post-vaccination have been depicted in *Table 2*. Two means of UVAS-CASTLE VAC and LaSota NDV are significantly different from each other even at day 0. In G1, maximum ELISA geometric mean antibody titers Log<sub>2</sub> was accomplished at day 14, e.g., 2380 as followed in G2, i.e., 1867, respectively. The geometric mean ELISA antibody titers Log<sub>2</sub> were significantly higher (P<0.05) in G1 as compared to G2. The results of geometric anti-NDV-HI antibody titers at 0, 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>th</sup>, and 28<sup>th</sup> days of post-vaccination have been presented in *Table 1*. Optimum geometric anti-NDV-HI antibody titers were observed in G1 at day 14, e.g. 7.5 as compared to G2 i.e. 6, respectively. These findings in contrast with the outcomes from the earlier studies, pheasants were vaccinated with commercially available NDV LaSota vaccine and humoral immune response was evaluated on the basis of agglutinin titer. Anti-NDV antibodies were found up to 75% of birds. Booster dose was given at 21<sup>th</sup> day of post vaccination, and then antibodies were depicted in all experimental birds<sup>[33]</sup>. Similar contrast findings were reported when ring-necked pheasants were vaccinated with different strains of Newcastle disease virus such as Ulster, B1 and LaSota. No maternally derived antibodies were detected at seven days of age. First protective antibodies response was observed at 10<sup>th</sup> day of post vaccination. Booster dose was given to maintain antibody titer against NDV up to 94 days of age.

The results of this project have shown that the use of UVAS-CASTLE VAC (Thermostable NDV I-2 strain) vaccine in pheasants is effective, immunogenic, economical and feasible, and leads to a protective immunogenic response. This may prevent the transmission of NDV into other birds especially poultry birds. Furthermore, administration of oral vaccine in feed to the same age free range pheasants overcomes the individual catching hurdles of these birds during vaccination.

**Table 1.** Comparative geometric mean anti-NDV-HI titer of G1 UVAS-CASTLE VAC (I-2 NDV strain), G2 LaSota NDV vaccine and G3 Negative control (PBS) in ring-necked Pheasant.

Group No.	Vaccination	Route	HI Mean Antibody titer (Days)				
			0	7	14	21	28
G1	I-2 NDV	D/W	3.8±0.62	6.6±0.64	7.6±0.75	6.2±0.61	4.4±0.59
G2	LaSota NDV	D/W	3.8±0.52	4.8±0.56	5±0.61	5.5±0.60	3.6±0.51
G3	NC (PBS)	D/W	3.8±0.52	2.8±0.48	2.2±0.41	1.8±0.34	1.2±0.30

The anti-NDV-HI titer pheasant vaccinated with NDV I-2 vaccine in G1 was significantly higher as compared to that pheasant G2 vaccinated with LaSota vaccine during 7 to 28 days post vaccination ( $P>0.05$ ); NDV (Newcastle disease virus); NC (Negative control); PBS (Phosphate buffer saline); D/W (Drinking water); G (Group); HI (Haemagglutination Inhibition test)

**Table 2.** Comparative geometric mean anti-NDV-ELISA titer of G1 UVAS-CASTLE VAC (I-2 NDV strain), G2 LaSota NDV vaccine and G3 Negative control (PBS) in ring-necked pheasant

Group No.	Vaccination	ELISA Mean Antibody titer (Days)				
		0	7	14	21	28
G1	I-2 NDV	1235±0.141	1796±0.346	2280±0.454	1960±0.379	1144±0.126
G2	LaSota NDV	1231±0.141	1523±0.237	1767±0.354	1680±0.335	1144±0.126
G3	Negative Control (PBS)	1231±0.141	1120±0.134	1061±0.097	1064±0.099	1079±0.111

The anti-NDV-ELISA titer pheasant vaccinated with NDV I-2 vaccine in G1 was significantly higher as compared to that pheasant G2 vaccinated with LaSota vaccine during 7 to 28 days post vaccination ( $P>0.05$ ); NDV (Newcastle disease virus); NC (Negative control); PBS (Phosphate buffer saline); D/W (Drinking water); G (Group); ELISA (Enzyme-linked immunosorbent assay)

## DISCUSSION

ND is one of the most pathogenic diseases which hampered the development of pheasant farming, if not successfully controlled. This includes the development of effective methods to combat this deadly disease. It was difficult to immunize the free-range or uncaged pheasants since conventional or old techniques were developed for the use in commercial and domestic birds. ND spread through contamination of feed and water with NDV infected droppings. Moreover, flying birds have the capability to transfer this virus among various species of birds and mainly responsible for many epidemics in the recent past [21]. Pheasants are carriers of virulent Newcastle disease virus strains. Hence, control of NDV in pheasant by vaccination is necessary to control or minimize the chances of the spread of ND in commercial birds [25,26]. It was therefore the objective of this project to prepare a thermostable NDV I-2 vaccine and evaluation of its efficacy in flying birds like pheasants. For this purpose, 8-9 days old chicken embryos were used to obtain maximum growth of NDV I-2 strain. Similarly, thermostable NDV I-2 virus has been grown in 8-day-old embryonic eggs in central laboratories in the developing countries. These embryonic eggs are not strictly pathogen-free, but are harvested from a healthy poultry flock that is routinely evaluated for main viral diseases such as ND [27]. Biological method i.e. egg infective dose fifty ( $EID_{50}$ ) for titration of NDV I-2 strain presented optimum titers  $10^9 EID_{50}/mL$  in the next 24 h of incubation, when grown in nine days old chicken embryos. Similar findings were reported when

thermostable NDV I-2 strain produced optimum titer  $10^9$  tissue culture infective dose 50 mL ( $TCID_{50}/mL$ ), after growing in Vero cell line [23]. In other recent studies, when NDV grown in 8-9 days embryonated eggs,  $10^8 EID_{50}/mL$  titer has been detected [28].

The protective antibody titer is an indication of the good quality of any vaccine [29]. The HI and ELISA tests were used to evaluate the antibody titer in pheasant birds following oral administration of the Thermostable NDV I-2 vaccine in this study. The results of both tests showed that optimum antibody titer was reached on day 14 after vaccination. Statistically, the highest mean protective antibody titers Log 2 was observed in G1 ( $P<0.05$ ) as contrasted to G2. In zoo birds, the antibody titer above  $\geq 2^3$  or 3  $\log_2$  is intended to be protective against virulent NDV in experimental or field trials of chickens [30]. This protective antibody titer may also be applied to wild birds such as pigeons and pheasants [31]. Similar consequences have been documented in feral pigeons [32] and guinea fowls when NDV I-2 strain vaccine was administered orally and mixed with feed. Vaccine administration route has significant effect on the production of protective antibodies titer. Eye drop administration produced maximum protective titer of antibodies as compared to other routes [33,34]. Ulster 2C NDV strain produced low antibody titer as compared to LaSota and B1 NDV strain [22]. Pheasants did not show any adverse effect after oral vaccination in the present study. It was concluded that the use of thermostable NDV vaccination techniques in wild birds, particularly pheasants, led to a reduction in the risk of transmitting of this horrible disease from these birds to poultry birds.



The present research concluded that the wild birds particularly pheasants have highlighted the spread of ND in commercial as well as rural poultry birds, therefore, they are significant risk factors for ND epidemics. Vaccination is the only way of preventing infectious diseases in the world. Commercially available NDV vaccines are usually thermolabile in nature and required a cold storage system to maintain their shelf life. In developing countries like Pakistan, where electric supply is a burning issue, it provides a viable and effective alternative to combat ND. In the future, we hope that the thermostable NDV I-2 vaccine will be much more widely used and implemented and help to manage Newcastle disease more effectively in the region.

#### ACKNOWLEDGEMENTS

Authors are immensely obliged to Higher Education Commission, Pakistan for their financial support in terms of IPFP start-up grant. Moreover, many thanks to workers and staff of Lahore, Safari Zoo supporting to conduct this study.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

#### AUTHORS CONTRIBUTIONS

F. Siddique, M. Rabbani, I. Hussain, have planned and designed the research. R. Z. Abbas, A. Iqbal, A. Rafique has contributed to the experimental method. R. Ahmad, M.S. Mahmood, A. Lotfi is helping to give the final form of the manuscript. All contributors discussed the findings and contributed to the final manuscript.

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