



## Experimental infection of pheasants with a velogenic chicken isolate of Newcastle disease virus

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### ABSTRACT

Newcastle disease (ND) is a highly contagious infection of many avian species, causing enormous losses in poultry production worldwide. The objective of this study was to reveal the clinical feature, virus shedding, and immune response following infection with a velogenic chicken isolate of Newcastle disease virus (NDV) in susceptible and vaccinated pheasants. Eighty day-old pheasant chicks were allotted to four groups. At 30 days of age, the birds in groups 1 and 3 were vaccinated with B1 strain via eye drop. Two weeks later, each bird in groups 1 and 2 was inoculated with 100  $\mu$ L (50  $\mu$ L/eye) of NDV-infected allantoic fluid containing  $10^5$  EID<sub>50</sub> of viral inoculum. All groups were inspected daily for three weeks. Swab samples were taken at different time points, and verified for NDV infection by using reverse-transcription polymerase chain reaction (RT-PCR). Serological examination was also made by haemagglutination-inhibition assay. Clinically, watery mucoid feces was observed only in one case among the vaccinated challenged birds, whereas the unvaccinated challenged birds showed anorexia, mild depression and head deviation. Out of 20 birds in group 2, one case (5%) died. Based on RT-PCR, virus shedding was only observed among the unvaccinated birds from 5 to 14 days after challenge. The NDV was detected more in tracheal swabs (40%) than in cloacal swabs (30%). The infected birds showed a high seroconversion. In conclusion, the velogenic NDV circulating in Iranian chicken flocks has a low pathogenicity for pheasants, and ocular vaccination with B1 strain could provide a good protection.

### Keywords

*Immune response, Newcastle disease, Pheasant, Virus shedding*

### Abbreviations

ND: Newcastle disease  
NDV: Newcastle disease virus  
EID<sub>50</sub>: 50% embryo infective dose  
HI: hemagglutination-inhibition  
RT-PCR: reverse-transcription polymerase chain reaction  
cDNA: complementary DNA  
bp: base pairs

## Introduction

Newcastle disease (ND) is a highly contagious and fatal disease affecting at least 241 species of domestic and wild birds of both sexes and all age groups [1]. The causative agent is a virulent virus of the avian paramyxovirus serotype I of *Avulavirus* genus belonging to the family *Paramyxoviridae* [2]. Velogenic Newcastle disease virus (NDV) is endemic in many countries of the Middle East, Africa and Asia [3]. The inclusion of ND in the list of notifiable diseases by World Organization for Animal Health (2008) is indicative of its paramount economic impact on the worldwide poultry industry [2]. Among poultry, chickens are the most susceptible, showing the most clinical signs, and ducks are the least susceptible, showing the least clinical signs. Nonvaccinated pheasants are highly susceptible with clinical signs similar to those observed in chickens [3].

The first outbreaks of ND were observed in chickens in 1926 in Java, Indonesia, and Newcastle-upon-Tyne, England [3]. Then, NDV was spread throughout the world, and affected other avian species such as turkeys and quails [4,5]. Several outbreaks of ND have been also reported in pheasants in East Anglia [6], Iraq [7], Great Britain [8], Denmark [9], and South East England [10].

In Iran, NDV is endemic in different parts of the country, causing enormous losses due to high mortality, sub-optimal production, slaughterhouse condemnation of carcasses, and high prevention and treatment expenses. In recent years, outbreaks of ND have been occasionally observed in different avian species in Iran, including Japanese quail [11], ostrich [12], exotic caged birds [13], and broiler chickens [14]. During 2012-2013, some outbreaks with heavy losses occurred among commercial broiler chicken flocks located in southwest Iran. The isolated viruses were classified as genotype VII, and subsequently to subgenotype VIIId [15].

Today, pheasant is extensively reared in several countries of the world as a game bird or for the purpose of human consumption. In recent years, commercial production of pheasant has increased in some regions of Iran, and a part of protein demands of Iranian people is provided with the meat of this bird. The NDV may be easily transmitted from one avian species to another, and some outbreaks in pheasants were epidemiologically related to the spread of the virus from chickens [6] and feral migratory birds [9]. Considering that viruses emerging from field strains may possess relatively new features, this study was conducted to investigate the clinical manifestations, virus shedding and serological responses following infection with a velogenic chicken isolate of NDV in

susceptible and vaccinated pheasants.

## Results

### Clinical signs

No morbidity or mortality was observed in unchallenged pheasants. The vaccinated challenged birds exhibited watery mucoid feces only in one case, whereas the unvaccinated challenged birds showed anorexia, mild depression and head deviation (Figures 1 and 2). Almost one-fourth of these birds became morbid, but 1 (5%) out of 20 pheasants died at 10 days postinoculation. Clinical symptoms appeared 7 days postinoculation and continued by 12 days after challenge.

### Virus shedding

An attempt to detect the virus was made for a period of three weeks, and the results of the PCR are presented in Figure 3. Out of 10 birds in group 2 which were sampled after challenge, 6 cases (60%) shed the NDV through respiratory and/or intestinal tracts from 5 to 14 days postinoculation. The NDV was detected more in tracheal swabs (40%) than in cloacal swabs (30%). The tracheal swabs were virus-positive at 5 and 10 days post-challenge, but the cloacal swabs were virus-positive at 10 and 14 days. The NDV was not detected in samples obtained from the other groups (Table 1).

### Serological examination

The antibody response of pheasants to vaccination and or challenge with velogenic NDV is summarized in Table 2. The HI titers of the serum samples of all groups were negative (i.e.,  $< \text{Log}_2 3$ ) before vaccination. This status continued in the birds of group 4 by the end of the experiment, whereas a significant seroconversion occurred after vaccination or challenge in the other groups ( $p < 0.05$ ). In group 3, the HI titer increased significantly after vaccination ( $p < 0.05$ ), but its change was not significant from 14 to 28 days post-vaccination ( $p > 0.05$ ). After challenge, the HI titers increased in groups 1 and 2; although its elevation was only significant ( $p > 0.05$ ) in group 2.

## Discussion

The NDV continues to be a major threat to the poultry industry. After infection with velogenic NDV, the nonvaccinated birds may die suddenly with no clinical signs and with a death rate of 100% [3], although the severity of the disease observed with any given virus greatly varies depending on host parameters, including species, breed, age and immune status,



**Figure 1**  
Depression in a susceptible pheasant chick inoculated with a velogenic chicken isolate of Newcastle disease virus (7 days postinoculation).



**Figure 2**  
Head deviation in a susceptible pheasant chick inoculated with a velogenic chicken isolate of Newcastle disease virus (8 days post-inoculation).



**Figure 3**  
Electrophoresis of RT-PCR product of F gene in pheasants inoculated with a velogenic chicken isolate of Newcastle disease virus; M: ladder (100 bp), N: negative control, P: positive control (330 bp), lanes 1 - 5: positive samples.

**Table 1**

Virus shedding in pheasants experimentally infected<sup>1</sup> with a velogenic chicken isolate of Newcastle disease virus.

| Group                         | Swab     | Days postinoculation <sup>2</sup> |   |   |    |    |    |
|-------------------------------|----------|-----------------------------------|---|---|----|----|----|
|                               |          | 0                                 | 2 | 5 | 10 | 14 | 21 |
| Vaccinated and challenged     | Tracheal | -                                 | - | - | -  | -  | -  |
|                               | Cloacal  | -                                 | - | - | -  | -  | -  |
| Unvaccinated and challenged   | Tracheal | -                                 | - | 2 | 2  | -  | -  |
|                               | Cloacal  | -                                 | - | - | 1  | 2  | -  |
| Vaccinated and unchallenged   | Tracheal | -                                 | - | - | -  | -  | -  |
|                               | Cloacal  | -                                 | - | - | -  | -  | -  |
| Unvaccinated and unchallenged | Tracheal | -                                 | - | - | -  | -  | -  |
|                               | Cloacal  | -                                 | - | - | -  | -  | -  |

<sup>1</sup>At 30 days of age, pheasants in vaccinated groups received B1 strain vaccine via eye-drop. Two weeks later, each bird in challenged groups was inoculated through ocular route with  $10^5$  EID<sub>50</sub> of viral inoculum.

<sup>2</sup>At each time point, two birds per group were sampled and examined for NDV infection by RT-PCR



**Table 2**  
Haemagglutination-inhibition titers (Log<sub>2</sub>)<sup>1</sup> in pheasants following vaccination with B1 strain and or challenge with a velogenic chicken isolate of Newcastle disease virus.

| Group                         | Days after vaccination  |                         |                         |
|-------------------------------|-------------------------|-------------------------|-------------------------|
|                               | 0 <sup>2</sup>          | 14 <sup>3</sup>         | 28 <sup>4</sup>         |
| Vaccinated and challenged     | 1.4 ± 1.06 <sup>c</sup> | 4.8 ± 0.71 <sup>b</sup> | 5.6 ± 0.74 <sup>b</sup> |
| Unvaccinated and challenged   | 1.3 ± 1.04 <sup>c</sup> | 1.1 ± 0.83 <sup>c</sup> | 7.1 ± 0.88 <sup>a</sup> |
| Vaccinated and unchallenged   | 1.3 ± 1.04 <sup>c</sup> | 4.5 ± 0.93 <sup>b</sup> | 4.8 ± 0.96 <sup>b</sup> |
| Unvaccinated and unchallenged | 1.8 ± 1.04 <sup>c</sup> | 1.3 ± 1.05 <sup>c</sup> | 0.9 ± 0.83 <sup>c</sup> |

<sup>a-c</sup> Values within columns/rows with no common superscripts differ significantly ( $p < 0.05$ ).

<sup>1</sup> Values represent Means ± SE calculated from eight birds per group at each time point.

<sup>2</sup> Thirty days of age, <sup>3</sup> Before challenge, <sup>4</sup> Two weeks after challenge.

coinfection with other organisms, environmental and nutritional conditions, and route of exposure [1,16]. The virus used in this study was isolated from an outbreak of ND in a vaccinated chicken flock with a high mortality, and was characterized as a velogenic strain based on the sequencing of the F protein cleavage site. However, in this experiment, only one of 20 birds in group 2 died and the other morbid birds recovered rapidly. To our knowledge, there is no information regarding the experimental pathogenicity of NDV for pheasants to be compared with our results. But, the lower severity of the disease observed in pheasants can be associated with their less susceptibility, as well as route of infection (i.e. experimental vs natural) [2,10]. In a similar study performed by Wakamatsu et al. (2006), chickens challenged with velogenic NDV exhibited clinical signs at 2 days after inoculation and experienced a mortality rate of 100%, whereas commercial turkeys developed the clinical disease later (i.e., 6 days after inoculation) with no mortality. [16]. In the ND outbreaks in Italy, Capua et al (2000) reported that chickens and Guinea fowl were the most affected species, followed by pheasants, turkeys and ostriches [17]. In the current study, the vaccination of pheasants with B1 strain did not cause any clinical signs associated with post-vaccinal reactions. Similarly, Schmidt et. al. (2008) didn't find any reactions in pheasants vaccinated via eye-drop with B1, Ulster 2C, or LaSota strains [18]. The general clinical signs, including anorexia, lethargy, and head deviation observed in the unvaccinated challenged birds are the typical of the disease. They were all reported previously in natural infections of pheasants with NDV [6,7,17,19].

RT-PCR is one of the reliable laboratory techniques facilitating a rapid diagnosis by detecting NDV virus in clinical specimens [2]. In the current study, NDV was first detected in tracheal swabs obtained from nonvaccinated challenged pheasants at 5 days postinoculation before the appearance of the clinical

signs (i.e., 7 days after inoculation). In our previous work, the Wishard bronze poulters experimentally infected with highly virulent NDV of chicken isolate shed the virus 2 days earlier than the exhibition of clinical signs [20]. Wakamatsu et al. (2006) isolated NDV from swab samples of infected turkeys at 2 days postinoculation, whereas the onset of the disease was at 6 days postinoculation [16]. Moreover, in the present study, the tracheal swabs had a higher virus detection rate (4/10), compared to the cloacal swabs (3/10). This is somewhat consistent with the

results reported by the previous studies, in which NDV was more frequently isolated from oral swabs than from cloacal swabs [16,21]. The detection or isolation rate of NDV may be influenced by the tropism of the virus. In an experimental study performed by Perozo et al. (2008), VG/GA strain of NDV was detected more in samples obtained from the intestinal tract of broiler chicks, whereas LaSota strain was detected more in samples taken from respiratory tracts [22]. Nevertheless, some studies have demonstrated the lack of sensitivity in detecting the virus in fecal samples, because they contain more extraneous organic material that can interfere with RNA recovery and amplification by PCR, suggesting that tracheal or oropharyngeal swabs are often the specimens of choice [2]. In the present study, virus shedding was not observed in vaccinated challenged pheasants. This is likely to be associated with less sensitivity of virus detection than isolation method, the small number of birds sampled at any time point after challenge, and or less susceptibility of pheasants to NDV in comparison with chickens, however additional studies are needed to clearly explain this finding.

As shown in Table 2, the HI titer in group 4 was lower than log<sub>2</sub>3 before vaccination, and remained constant during the experiment, which can be regarded as being nonspecific [2]. On the other hand, a sudden seroconversion was observed in the other groups, implying the induction of active immunity by vaccinal or challenging virus. In group 3, mean HI titers of 4.5-4.8 (Log<sub>2</sub>) were resulted from vaccination, but did not change significantly during 14 to 28 days postvaccination. In a similar study performed by Schmidt et. al. (2008), ocular vaccination of 10-day-old pheasants with B1, Ulster 2C, or LaSota produced mean HI titers of 4.2-5.0 (Log<sub>2</sub>) at 24 days of age [18]. In group 2, a higher HI titer (7.1) was found after challenge by velogenic NDV. Aldous et al. (2007) reported HI titers of 2<sup>4</sup>-2<sup>8</sup> (tested with 8 HAU of antigen) in pheasants suffering from an outbreak of ND [10]. Piacenti et al.

(2006) found a significant seroconversion at 10 days after infection of velogenic NDV in commercial turkeys [23]. In group 1, challenge with velogenic NDV could not make a significant rise in the HI titer, which may be associated with the interference of active antibodies with velogenic NDV. Alexander and Senne (2008) reported that eye-drop vaccination of chickens with Hitchner B1 will result in production of lachrymal IgM, IgG, and IgA due to the replication of virus in the Harderian gland, which could be prevented by the presence of maternal IgG in lachrymal fluid [24]. These findings indicate that serum antibody alteration in pheasants following exposure to NDV is rapid and very similar to that in chickens and turkeys. In infection of chickens with NDV, antibodies usually are detectable in the serum within 6-10 days and reach the peak after 2-4 weeks [22,24].

In conclusion, the results indicated that the velogenic NDV circulating in Iranian chicken flocks has a low pathogenicity for pheasants, and that ocular vaccination with B1 strain along with biosecurity could provide a good protection.

## Material and methods

The velogenic NDV used in this experiment was isolated from a broiler chicken flock in southwest Iran during an outbreak in 2013. Based on nucleotide sequence, the virus was previously characterized as genotype VII (subgenotype VIIId), and assigned an accession number of NDa:KP347437 [15]. Initially, the virus was propagated twice in 9-day-old embryonated chicken eggs through inoculation into chorioallantoic sac. The 50% embryo infective dose ( $EID_{50}$ ) was calculated for the second passage according to the method of Reed and Muench [25], and the harvested allantoic fluid was used as inoculum as specified in the experimental design.

## Experimental Design

A total of 80 day-old unsexed pheasant chicks were purchased and randomly assigned into four equal groups. They were housed in cages separately in the Animal Research Unit of Shahid Chamran University of Ahvaz, and received feed and water ad libitum during the experiment. At 30 days of age, when the sera were negative for maternal antibodies in conventional hemagglutination-inhibition (HI) test, the birds in groups 1 and 3 were vaccinated with live B1 strain of NDV via eye drop; but those in groups 2 and 4 were sham-vaccinated with distilled water. Two weeks later, each bird in groups 1 and 2 was inoculated with 100  $\mu$ L (50  $\mu$ L/eye) of NDV-infected allantoic fluid containing  $10^5$   $EID_{50}$  of viral inoculum, whereas the birds in groups 3 and 4 received distilled water by the same route. All birds were daily inspected for clinical manifestations and mortality for three weeks.

## Sample collection

Tracheal and cloacal swabs were obtained from two birds per group before inoculation and at 2, 5, 10, 14, and 21 days postinoculation. They were examined for NDV infection by reverse-transcription polymerase chain reaction (RT-PCR). Moreover, eight birds from each group were bled through jugular vein before vaccination and at 14, and 28 days postvaccination. The blood samples were left to coagulate at room temperature for 8 h, and then

centrifuged at 2000 rpm for 5 min. The collected sera were stored at  $-20^{\circ}\text{C}$  until examined by HI test.

## Reverse-transcription polymerase chain reaction

The swab samples were individually placed in microtubes containing 250  $\mu$ L phosphate-buffered saline (PBS). After removing them from the microtubes, the RNA extraction was performed using the RNXTM-Plus Kit (CinaGen, Tehran, Iran) according to the manufacturer's protocol. The isolated RNAs were directly used for the RT-PCR or stored at  $-70^{\circ}\text{C}$ . The partial F gene, including the cleavage site sequence, was amplified using a pair of specific primers. The primer sequences were TT GAT GGC AGG CCT CTT GC and GG AGG ATG TTG GCA GCA TT [14]. The complementary DNA (cDNA) was synthesized using BioNeer RT PreMix kit (BioNeer Corporation, South Korea) according to the manufacturer's instruction. The RT-PCR assay was carried out in a 20  $\mu$ L reaction volume consisting of 2  $\mu$ L of 10x PCR buffer, 0.2  $\mu$ L of 10 mM dNTPs, 1  $\mu$ L of each primer (20 pmol/ml), 0.2  $\mu$ L Taq DNA polymerase (5U/ml), 0.6  $\mu$ L of 50 mM magnesium chloride, 10  $\mu$ L distilled water, and 5  $\mu$ L cDNA dilution. The RT-PCR conditions included initial denaturation at  $95^{\circ}\text{C}$  for 3 min, followed by 40 cycles of  $95^{\circ}\text{C}$  for 30 sec,  $55^{\circ}\text{C}$  for 60 sec,  $72^{\circ}\text{C}$  for 60 sec, and a final extension at  $72^{\circ}\text{C}$  for 10 min. The RT-PCR products with 330 base pairs (bp) were subjected to electrophoresis using 1.5% agarose gel. The NDV-infected allantoic fluid from our previous work was used as positive control, and distilled water was employed as negative control. In addition, a 100-bp DNA marker was used in electrophoresis for determining the RT-PCR product size.

## Haemagglutination-inhibition test

The sera obtained by centrifugation of the samples were left in a water bath at  $56^{\circ}\text{C}$  for 30 min. Then, they were assessed for haemagglutination-inhibiting antibodies using 4 HA units of NDV antigen and two-fold serum dilutions as recommended by Thayer and Beard (2008) [26]. The results were expressed as Log<sub>2</sub>.

## Statistical analysis

All data were analyzed in SPSS software (Version 24.0., Armonk, NY: IBM Corp.) using one-way analysis of variance. Differences showing  $p < 0.05$  were considered statistically significant [27].

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## Author Contributions

Conceived and designed the experiment: RAJ, AR, ZB, Consulted: MM, Performed the experiment: RAJ, AR, ZB, RZ, Wrote the paper: RAJ, RZ.

## Conflict of Interest

The authors declare that they have no conflicts of interest.

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## عفونت تجربی قرقاول ها با ویروس حاد نیوکاسل جدا شده از ماکیان گوشتی

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### چکیده

بیماری نیوکاسل یک عفونت واگیردار در بسیاری از گونه های پرندگان است که خسارت زیادی به صنعت طیور دنیا وارد می کند. این مطالعه با هدف تعیین چهره بالینی، دفع ویروس و پاسخ ایمنی به دنبال آلودگی با ویروس حاد نیوکاسل جدا شده از ماکیان گوشتی در قرقاول های حساس و واکسینه انجام گرفت. هشتاد جوجه قرقاول یکروزه به ۴ گروه تقسیم شدند. در ۳۰ روزگی، گروه های ۱ و ۳ با واکسن B1 به روش قطره چشمی واکسینه شدند. دو هفته بعد، هر پرنده در گروه های ۱ و ۲ با 105EID50 (number 5 have to be) (in superscript) ویروس نیوکاسل در حجم ۱۰۰ میکرولیتر (۵۰ میکرولیتر در هر چشم) چالش شد. تمام گروه ها برای ۳ هفته زیر نظر بودند. نمونه های سوآب در مقاطع زمانی مختلف تهیه و از نظر آلودگی به ویروس نیوکاسل به وسیله واکنش زنجیره ای پلیمرز معکوس (RT-PCR) بررسی گردیدند. پاسخ ایمنی نیز با آزمایش ممانعت از هم‌آگلوتیناسیون بررسی شد. قرقاول های گروه ۱ فقط در یک مورد مدفوع موکوسی آبکی داشتند. قرقاول های گروه ۲ نشانه های بی اشتها، افسردگی و انحراف سر را بروز دادند. از ۲۰ پرنده در گروه ۲، یک مورد (۵ درصد) تلف شد. بر اساس RT-PCR، دفع ویروس نیوکاسل فقط در قرقاول های غیرواکسینه و در روزهای ۵ تا ۱۴ بعد از چالش مشاهده گردید. میزان ردیابی ویروس به وسیله سوآب نای (۴۰ درصد) بیش تر از سوآب کلواک (۳۰ درصد) بود. تغییرات سرولوژیک در قرقاول های آلوده نیز معنی دار بود. نتیجه گیری می شود که ویروس حاد نیوکاسل موجود در گله های ماکیان ایران، بیماری زایی کمی برای قرقاول ها دارد و واکسن قطره چشمی B1 می تواند محافظت خوبی ایجاد کند.

### واژگان کلیدی

پاسخ ایمنی، بیماری نیوکاسل، قرقاول، دفع ویروس