

## **Determination of median embryo lethal dose for a velogenic Newcastle disease virus isolated in Sulaimani/Iraq**

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### **Abstract**

Newcastle disease (ND) is an endemic viral illness in Iraq and has four types: viscerotropic velogenic, neurotropic velogenic, mesogenic, and lentogenic. The virulence of the virus continuously increases, and it is widely spread in wild and domestic birds. Backyard and poultry farm chickens are mainly affected, causing significant economic losses, as the disease is famous for high morbidity and mortality, which may reach 100%. An outbreak of velogenic ND spread in poultry farms in Sulaymaniyah/Iraq in early 2023, causing high mortality rates. Hence, this study was conducted to isolate and identify the virus and determine its median embryo lethal dose (ELD50) in embryonated chicken eggs (ECEs). Samples were taken from chickens suspected of ND, and NDV identification was made by molecular techniques by amplifying part of the 535-base pair-F gene. The virus was passaged three times in ECEs, and the collected allantoic fluid was used to determine the ELD50. After that, fifty ECEs were used to calculate the ELD50. Allantoic fluid dilutions of 10–107 resulted in the death of all embryos after 2-3 days, with distinctive signs of bleeding and hemorrhage. Dilutions of 108, 109, and 1010 resulted in the death of four, two, and one embryo, respectively, and the PCR test revealed NDV infection. The ELD50 was  $6.3 \times 10^8$  times the allantoic fluid dilution, showing that the virus was very velogenic and necessitated a strict control plan to prevent the disease's further spread.

**Key words:** Newcastle disease, ELD50, eggs, PCR.

## Introduction

ND was first reported about a century ago in Newcastle-upon-Tyne, England, and Java, Indonesia, and the first descriptions of the disease were in 1926 (1). However, it remains an epizootic in Asia, Africa, and Central and South America, and intermittent epizootics occur in Europe (2). Cormorants, pigeons, and imported psittacine species are the most frequent carriers of the virulent Newcastle disease virus (NDV). In addition, poultry and wild birds, particularly ducks, frequently have low virulent NDV strains, which reduce productivity (3). A disease with an enormous economic impact, ND presents a severe danger to the poultry industry (4). It is endemic in many developing nations and primarily affects areas where people depend on raising poultry for a living (5). The NDV that attacks poultry shows manifestations of gastrointestinal, respiratory, and neurological disorders and causes deaths of up to 100% depending on the viral pathotype (6). The annual losses brought on by this illness are estimated in millions of dollars (7). Variable NDV strains cause different death rates and sickness in a flock (8), which also causes a decline in egg production (9). Because it is inexpensive and contains high-quality protein, poultry meat is a crucial source of protein in people's diets. However, meat quality in most developing countries may be affected by ND infection (10).

The NDV, belonging to the Paramyxoviridae family and subfamily Avulavirinae, was formally renamed avian avulavirus 1 by the International Committee on Taxonomy of Viruses (ICTV) in 2016 and again as avian orthoavulavirus 1 in 2018 (11). Virions are enveloped, pleomorphic particles varying in diameter from 100 to 500 nm and weighing about 15.2 kb (12). NDV is an enveloped, linear, non-segmented, and negative-sense

single-stranded RNA virus (13) with a genome containing six genes with 15,186 nucleotides. These genes include nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) protein, and large protein (L) 3'-NP-P-M-F-HN-L-5' (13).

Signs of infected birds vary depending on the strain of the virus, bird species, concurrent disease, and preexisting immunity (7). According to virulence, the Newcastle disease virus is divided into four types. The viscerotropic velogenic type occurs as depression, inappetence, substantial drop in egg production, increased respiration, a profuse greenish-yellow diarrhea rapidly leading to dehydration and collapse, swollen heads, and cyanotic combs. Mortality can be up to 90%, and infected birds usually die within one or two days. Birds that survive the initial phase develop nervous signs usually. Sometimes birds die without previous clinical signs (3).

In the neurotropic velogenic ND, acute signs from the respiratory tract and nervous system dominate. Sudden depression, inappetence, and a drop in egg production are seen together with coughing and other signs from the respiratory tract, followed by nervous signs within a few days. Mortality is usually around 10-20% for adult birds but can be higher for young birds (3).

Coughing and other signs from the respiratory tract dominate in the mesogenic ND. Other symptoms are depression, weight loss, and decreased egg production for up to three weeks. Signs from the nervous system can develop late in the disease. Mortality is around 10% (14). Lentogenic NDVs are often subclinical, but mild respiratory signs and a slight drop in egg production can be seen. The

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disease has no nervous signs, and mortality is usually negligible (3).

ND is endemic in Iraq, and outbreaks occur continuously. A new outbreak occurred in Sulaimaniyah/Iraq, in 2023, causing mortality rates of up to 80% in the affected poultry houses. Accordingly, this study determined the virus' degree of pathogenicity by isolating the virus in embryonated chicken eggs and determining the median embryo lethal dose (ELD<sub>50</sub>).

## Materials and Methods

### ND Detection of and confirmation:

Diseased broilers with signs of NDV were brought to Biolab Veterinary Clinic in Sulaimaniyah/Iraq. The chicks had clinical signs suggestive of ND, like hemorrhage of the proventriculus and cecal tonsils, tremors, and paralyzed wings. Samples from the brain, liver, cecal tonsils, lungs, tracheas, and respiratory secretions were collected aseptically from 3–5 diseased birds. The samples were put in a Petri dish, the solid parts were ground by a bladder, and a small pestle did complete grinding.

RNA extraction was done using Total RNA Mini Kit (Tissue) developed by AddBio-Korea. All manufacturer-recommended protocols were followed.

In this study, the ND vaccine was used as a positive control, and it was extracted like the samples to validate the extraction procedure and the PCR amplification. Additionally, distilled water was extracted alongside the sample to serve as a negative control and to detect contamination throughout the process.

Macrogen® (Korea) produced the primers used in the study. The clinical signs of ND are

close to those of avian influenza (AI) and infectious bronchitis (IB), which may sometimes lead to misdiagnosis. Mixed infections with NDV and these viruses are also not uncommon.

Hence, the samples were also tested for AI and IB viruses using appropriate primers to exclude mixed infection and misdiagnosis. The primer sequences are illustrated in Table 1.

A one-step RT-PCR was used to amplify the fusion (F) gene for NDV, the S1 gene for IBV, and the M gene for AIV. The reaction was carried out in 0.2 mL PCR tubes containing 10 µL master mix, 5 µL RNA, and 1 µL (10 pmol) of each forward and reverse primer (Table 2). Macrogen, South Korea, manufactured the primers. The reaction volume was completed to 20 µL by adding 3 µL of Q water.

The PCR tubes were put in the thermocycler and programmed to run the samples using different protocols according to the virus type. The samples were processed to diagnose three viruses common in poultry in Sulaimani province: NDV, AIV, and IBV. The duration and temperature of each cycle are illustrated in table 3.

The PCR products were run on an agarose gel to observe the gene sequence amplicon. The PCR product was visualized by illuminating the gel with UV light using the SafeBlue Illuminator/Electrophoresis system, and a digital camera documented the PCR bands. The bands were compared with the 100-bp DNA ladder.

**Table 1. Primer sequences of the tested viruses**

Virus	Gene name	Amplicon size (bp)	Primer	Sequence (5'-3')	Reference
NDV	F	535	F	ATGGGCYCCAGACYCTTCTAC	(15)
			R	CTGCCACTGCTAGTTGTGATAATCC	
IBV	S1	448	F	GTT TAC TACTAC CAA AGT GCC TT	(16)
			R	GTG TAA ACA AGG TCA CCA TTT A	
AIV	Matrix (M)	244	F	ATGAGYCTTYTAACCGAGGTTCGAAACG	(17)
			R	TGGACAAANCGTCTACGCTGCAG	

F = forward; R = reverse; bp = base pair.

**Table 2. PCR tube content**

Compound	Amount ( $\mu$ L)		
	NDV	IBV	AIV
One-step RT-master mix	10	10	10
Gene content (sample)	5	5	5
Primers	forward	1	1
	reverse	1	1
Q water	3	3	3
Total content in the PCR tube	20	20	20

**Table 3. Times for different stages of PCR**

Virus	Stage	Temperature ( $^{\circ}$ C)	Time (seconds)	No. of cycles
NDV	Pre-denaturation	95	300	1
	Denaturation	95	30	
	Annealing	57	30	55
	Extension	72	40	
	Final extension	72	300	1
	Pre-denaturation	95	300	1
IBV	Denaturation	95	30	
	Annealing	58	30	56
	Extension	72	40	
	Final extension	72	300	1
	Pre-denaturation	95	300	1
AIV	Denaturation	95	30	
	Annealing	56	30	54
	Extension	72	40	
	Final extension	72	300	1

### **Embryonated chicken egg inoculation by NDV and vaccine**

The tracheal swab samples were put into a viral transport medium (VTM) containing phosphate-buffered saline (PBS), penicillin G (10,000 IU/mL), streptomycin (10 mg/mL), and amphotericin B (250 IU/mL). The components were homogenized to create a 10% w/v suspension in a tryptose phosphate broth (TPB) solution, pH 7.0–7.2. Materials that had been homogenized were spun at 1000 g for 15 minutes to allow the accumulating bacteria and fungi at the bottom of the tube. Then, the supernatant was taken and recentrifuged for about five minutes to obtain a clear viral suspension.

Fertilized chicken eggs (not vaccinated for ND) were incubated at 37.5–39.5°C and 60% humidity. Three ECEs were injected with 0.1 mL sterile normal saline and were incubated until the end of the incubation period (21 days) to ensure that the injection technique did not kill or injure the embryos. After it was confirmed that the injection technique did not harm the embryos, the next step was to use an ND vaccine to inoculate ECEs. The eggs were inoculated with an ND vaccine in the first trial to be sure of our technique and show the signs of ND infection in chick embryos.

After mastering the egg inoculation technique using an ND vaccine, the live NDV isolated from infected broilers was used to infect embryonated chicken eggs. The inoculation was done on the ninth day of incubation by making a circle around the air sac, and the allantoic fluid was found by candling. Next, an 18-gauge needle made a hole in the eggshell at the air sac region. Then, 0.1 mL of the vaccine was inoculated into the egg using an insulin syringe, and the hole was sealed with wax. After that, the egg was reincubated for another day, and the embryo was looked at

by candling. Then, the egg was put into the refrigerator for one day for regression of the blood inside the embryo, and taking the allantoic fluid with ease. Then, the allantoic fluid was collected using a sterile syringe and centrifuged for 10 minutes at 1000 rpm to get a clear fluid containing the NDV. The virus was passaged three times in ECEs, five eggs per passage, from nonvaccinated chickens to ensure their infectivity. The collected allantoic fluid from each passage was tested for NDV using PCR.

### **Calculation of median embryo lethal dose or ELD<sub>50</sub>**

The ELD<sub>50</sub> of the previously isolated NDV was calculated using the method described by SE Grimes (18). The details of the procedure are described in the following sections.

The NDV-containing allantoic fluid collected from infected eggs was diluted in a ten-fold serial manner, covering a range from 10<sup>-1</sup> to 10<sup>-10</sup>. First, sterile tubes of 1.5 mL capacity were labeled according to the dilution factor (Figure 1). Then, 0.9 mL of VTM was added to each tube. After that, 0.1 mL of the allantoic fluid was put in the first tube and shaken thoroughly to mix the content. Then, 0.1 mL of the first tube's content was taken using a new pipette tip and put into the second tube, which was shaken to mix well. This procedure was repeated until the end of the ten tubes. By this, different dilutions from 10<sup>1</sup>–10<sup>10</sup> were prepared.

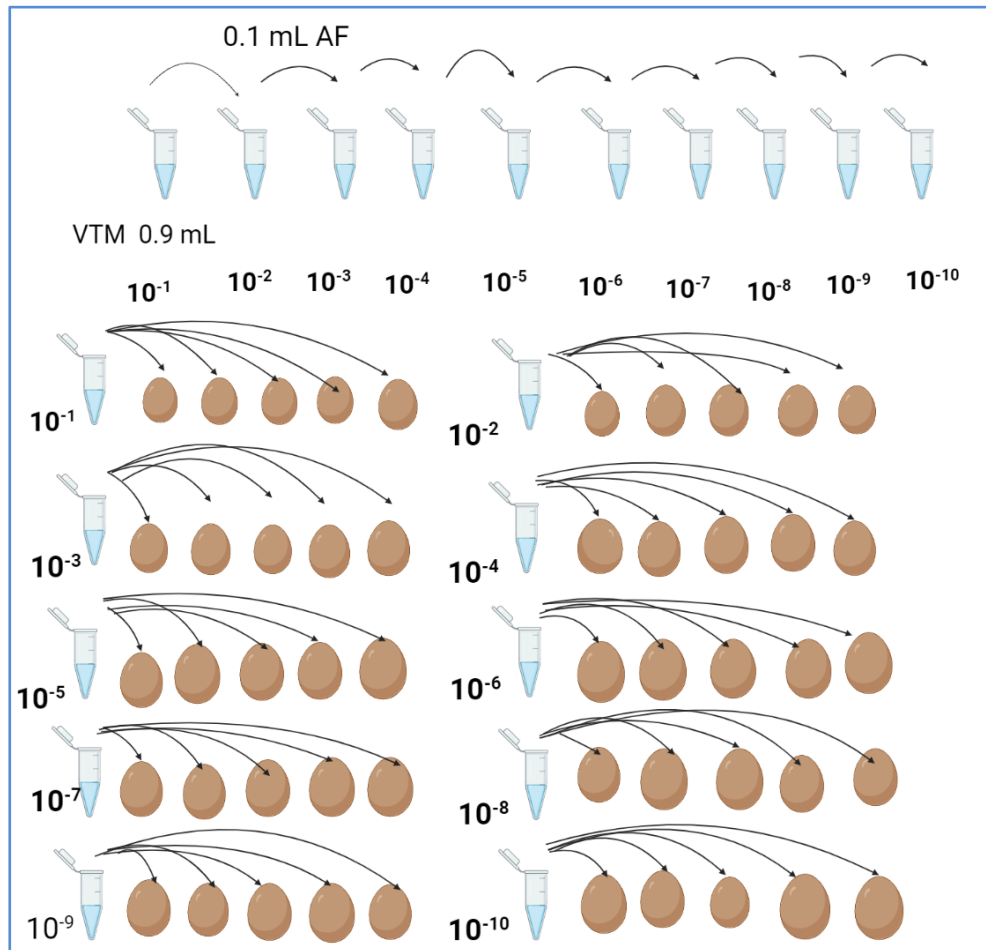
Fifty ECEs were used to calculate the ELD<sub>50</sub>. First, the eggs were divided into ten groups and labeled accordingly. Next, the eggs in each group containing nine-day-old embryos were injected with 0.1 mL of the diluted virus prepared in the previous step. After that, the virus was injected into the chorioallantoic fluid (CAF) using a sterile insulin syringe

with candling. Next, the eggs were incubated and checked daily to determine whether the embryo was alive or dead. If the embryo was dead, the egg was put in a refrigerator for 24 hours, and the allantoic fluid was collected. Then, the egg was cracked open to take out the embryo.

The number of dead embryos in each dilution group was recorded until the end of the incubation period. After that, a table was made to record the number of dead and live embryos, the accumulated number of dead

and live embryos, and the percentage of dead embryos in each dilution group after five days of incubation. Then, the following formula was used to find the index, which is a number that will be added to the dilution factor that produces mortality directly above 50%:

$$\text{Index} = \frac{\% \text{ dead at dilution immediately above } 50\% - 50\%}{\% \text{ dead at dilution immediately above } 50\% - \% \text{ dead at dilution immediately below } 50\%}$$



**Figure 1. Calculation of ELD<sub>50</sub>.** Different concentrations of NDV were prepared by conducting ten-fold serial dilutions of the CAF collected from an experimentally infected ECE. The dilution was conducted by adding 0.9 mL VTM to 0.1 mL CAF. Then, ten groups of eggs (five each) were inoculated with 0.1 mL of the diluted CAF. The eggs were then incubated for four days, and the number of dead embryos in each dilution was recorded. This percentage was used to calculate the ELD<sub>50</sub>.

### **Detection of ND in Naturally Infected Chicks**

The NDV in this study was isolated naturally from infected broilers at the poultry houses, and it was a viscerotropic velogenic virus because the mortality in the poultry houses was about 700–800 chickens a day. However, clinical signs of ND may be mixed with other diseases, such as IB and AI. So, for confirmation, PCR was done, and we confirmed and detected that the chicks were solely infected with NDV.

The samples collected from suspected chicks were confirmed for infection with NDV by PCR and visualization of the 535 bp gene fragment of the F gene (Figure 2). On the other hand, the samples were confirmed to be clear of AIV as the PCR and electrophoresis could not detect the 244 bp gene fragment of the virus's M gene. Also, the samples were clear for the 448 bp fragment of the S1 gene of IBV (Figure 3). So, the chicks were only infected with NDV and were free from AIV and ABV. Accordingly, these chicks were used as the source of NDV for isolating and propagating the virus in ECEs.

### **Egg Inoculation and Calculation of Median Embryo Lethal Dose**

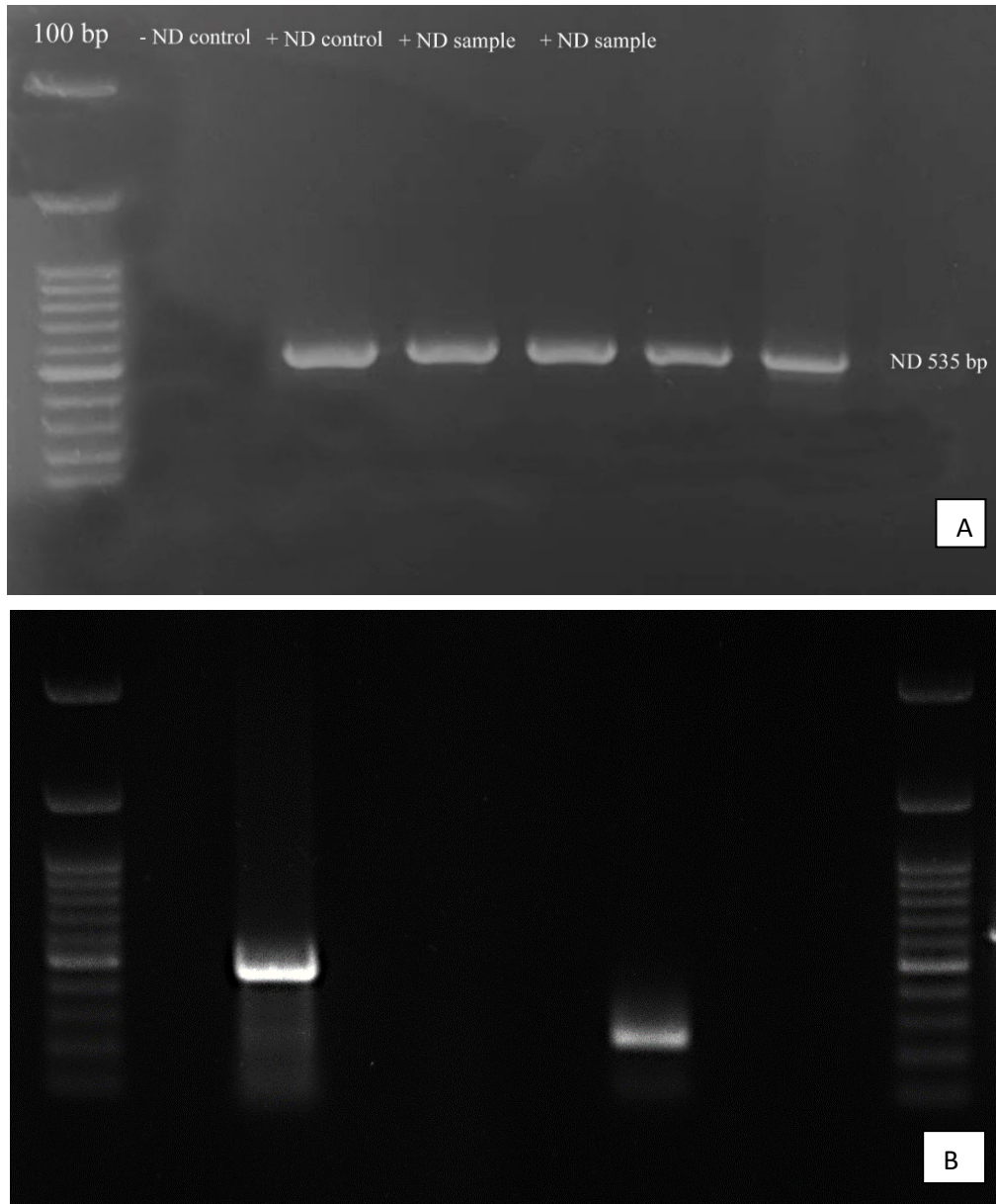
The normal saline-inoculated embryonated chicken eggs remained alive until the eggs

hatched after 12 days of inoculation (21 days of incubation), confirming the proper technique. Signs of ND were evident in embryos inoculated with the ND vaccine. It was noticed that the embryos stayed alive for 3 to 4 days, checked daily by candling. After that, the egg was broken, signs of hemorrhage were noticed on the embryos, and the color of one embryo was changed to black (Figure 3).

The embryos inoculated with the virus that naturally infected live chicks died after two to three days. Signs of hemorrhage were clear on the embryos, and the PCR test was positive for NDV and did not detect AIV or IBV. However, three passages were conducted to confirm the virus' infectivity and adaptation for inoculation of ECEs. Inoculation of embryonated eggs with diluted allantoic fluid caused the death of the embryos with 10<sup>-1</sup> to 10<sup>-7</sup> dilutions after 48 hours, and signs of hemorrhage were obvious in embryos. Also, dilution of 10<sup>-8</sup> caused the death of four embryos, 10<sup>-9</sup> caused the death of two embryos, and 10<sup>-10</sup> caused the death of one embryo after four days of inoculation. Signs of hemorrhage were evident on all dead embryos, and the PCR test confirmed infection with NDV. Then, the index was calculated as the following:

I

$$andex = \frac{\% \text{ dead at dilution immediately above } 50\% - 50\%}{\% \text{ dead at dilution immediately above } 50\% - \% \text{ dead at dilution immediately below } 50\%} = \frac{87.5 - 50}{87.5 - 42.9} = \frac{37.5}{44.6} = 0.84$$



**Figure 2. A:** Gel electrophoresis image for a sample infected with NDV, showing the 535 bp fragment of the F gene. **B:** Gel electrophoresis image for a sample with negative results for IBV and AIV. Lane L = 100 bp DNA ladder; Lane N1 = negative control for IBV; Lane P1 = positive control for IBV; Lanes 1 and 2 = negative result for IBV; Lane N2 = negative control for AIV; Lane P2 = positive control for AIV; Lanes 3 and 4 = negative result for AIV.





**Figure 3. Signs of NDV on chicken embryos. A: A black-colored embryo on day 12 of incubation, three days post-NDV-inoculation. B: Dead embryo examination. C: Hemorrhagic yolk sac blood vessels of an embryo. D: Hemorrhagic spots on an embryo died of ND infection.**

The percentages of dead embryos by different dilutions of allantoic fluid are shown in **Table 4**. The index was applied to the dilution that produced the percentage dead immediately

above 50%, which was  $10^{-8}$ . The index of 0.84 was applied to this dilution, and the  $ELD_{50}$  was  $10^{-8.84} = 6.3 \times 10^8$  times dilution.

**Table 4. Number of dead embryos four days after inoculation with different NDV dilutions**

Dilution	No. of dead embryos	No. of live embryos	Accumulated no.			Percentage infected
			Dead	Live	Total	
10 <sup>-7</sup>	5	0	12	0	12	100
10 <sup>-8</sup>	4	1	7	1	8	87.5
10 <sup>-9</sup>	2	3	3	4	7	42.9
10 <sup>-10</sup>	1	4	1	8	9	11.1

## Discussion

The NDV in this study was isolated naturally from infected broilers at the poultry houses, and it was a viscerotropic velogenic virus because the mortality in the poultry houses was about 700–800 chickens a day. Preparation of virus stocks by growth in embryonated eggs is an old and reliable method of producing stocks of many different types of viruses, such as NDV, and is, by far, the superior method for producing high-titer NDV stocks. This method can produce stocks of infectious viruses regardless of the virus strain (19).

NDV is an avian virus that has been extensively researched as an oncolytic virus, with a long history of clinical trials for this application (20). Due to host range restriction, it is not pathogenic in humans, which avoids the issue of preexisting immunity in the population (21). As such, it is an ideal candidate for a vaccine vector in terms of safety and immunogenicity and has been implemented in several studies targeting human and veterinary diseases (22). These vaccine candidates rely on the well-established production process in ECEs (23).

The virus propagation technique was confirmed when ECEs were inoculated with normal saline and embryos stayed alive. After that, by inoculating ECEs with the ND vaccine, signs of hemorrhage and quick death of embryos were helpful for the reorganization of NDV signs, which were very obvious in ECEs inoculated with a virus from naturally infected chicken. It was demonstrated that NDV results in embryonic demise, hemorrhage throughout the body, blood-filled subcutaneous tissues in the skull, and visible blood vessels all over the body. Another study, however, suggested that virulent NDV strains might not always kill the infected embryos (24), whereas all NDV-infected ones showed healthy embryos and hemorrhage (25).

At the beginning of the experiment, it was necessary to determine the ELD<sub>50</sub> in the ECEs, as the virus strain was velogenic and the median infective dose was not helpful. The ELD<sub>50</sub> is recommended for infecting live chicks with velogenic NDVs (24). Since the virus was velogenic, the embryos died quickly between 24 to 48 hours. Other researchers

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reported that velogenic NDV caused embryonic death on the second day of infection (26).

The results showed that infecting ECEs caused embryonic death on the second day, consistent with Balachandran *et al.* (27), that NDV strains caused high mortality in ECEs within 60–90 hours. However, Mulisa and coworkers reported that a velogenic NDV isolate caused embryonic death 3–6 days postinfection (27), meaning the NDV was less virulent than our study's.

The virus caused embryonic death within 60–100 hours. A study conducted in Iraq revealed that the ELD<sub>50</sub>, which resulted in the death of embryos in 24–96 hours, was 10<sup>6</sup> times dilution of the allantoic fluid (28). However, the ELD<sub>50</sub> of our experiment was a concentration of 6.3 × 10<sup>8</sup>, which means that the virus was more virulent than in the previous study, so it is essential to control the disease by putting boundaries and not allowing the transport of poultry randomly, especially in the backyard and exotic birds in our region. Mandatory sanitary rules in poultry houses should be applied, and regular vaccination may decrease morbidity and mortality.

## Conclusion

Newcastle disease virus is endemic and causes high economic losses in Iraq. In recent years the virus' virulence increased, and the rate of hazards continuously increased, meaning that vaccination and continuous efforts to control the disease were unsuccessful. Hence, a strategic plan and hard work are imperative to control and decrease the disease burden on poultry farming in Iraq.

**Conflict of interest:** All authors declare that there is no conflict of interest.

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## تحديد الجرعة المتوسطة المميتة للجنين لفيروس مرض نيوكاسل التطوري المعزول في السليمانية/العراق

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<sup>3</sup> فرع الاحياء المجهرية، كلية الطب البيطري، جامعة السليمانية، السليمانية، العراق.

### الخلاصة

مرض نيوكاسل هو مرض فيروسي متوطن في العراق وله أربعة أنواع: نضجي المنشأ، نضجي المنشأ العصبي، ميسوجينيك، ولين المنشأ. وتزداد ضراوة الفيروس بشكل مستمر، وينتشر على نطاق واسع في الطيور البرية والداجنة. ويتأثر بشكل رئيسي دجاج الفناء الخلفي ومزارع الدواجن، مما يسبب خسائر اقتصادية كبيرة، حيث يشتهر المرض بارتفاع معدلات الإصابة والوفيات التي قد تصل إلى 100%. انتشر تفشي مرض ND في مزارع الدواجن في السليمانية / العراق في أوائل عام 2023، مما تسبب في ارتفاع معدلات الوفيات. ومن ثم، أجريت هذه الدراسة لعزل وتحديد الفيروس وتحديد الجرعة المميتة المتوسطة للجنين (ELD<sub>50</sub>) في أجنة بيض الدجاج (ECEs). تم أخذ عينات من الدجاج المشتبه بإصابته بمرض نيوكاسل، وتم التعرف على الفيروس بواسطة التقنيات الجزيئية عن طريق تضخيم جزء من F (535 bp). تم تمرير الفيروس ثلاث مرات في ECEs، وتم استخدام السائل السقاء الذي تم جمعه لتحديد ELD<sub>50</sub>. بعد ذلك، تم استخدام خمسين ECEs لحساب ELD<sub>50</sub>. أدت تخفيفات السائل السقاء بمقدار 10<sup>7</sup>-10<sup>10</sup> إلى موت جميع الأجنة بعد 2-3 أيام، مع وجود علامات مميزة للنزف والنزف. وأدت التخفيفات 10<sup>8</sup>، و10<sup>9</sup>، و10<sup>10</sup> إلى وفاة أربعة، واثنين، وجنين واحد، على التوالي، وكشف اختبار PCR عن الإصابة بفيروس NDV. كان ELD<sub>50</sub> 6.3 × 10<sup>8</sup> أضعاف تخفيف السائل السقاء، مما يدل على أن الفيروس كان نشوياً للغاية ويستلزم خطة مراقبة صارمة لمنع انتشار المرض بشكل أكبر.

**الكلمات المفتاحية:** مرض نيوكاسل، الجرعة المميتة المتوسطة للجنين، بيض، تفاعل البلعمة المتسلسل.