The PHILIPPINE JOURNAL OF Veterinary Medicine

(Short Communication)

Isolation and Identification of Newcastle Disease Virus from Commercial Layers Farm with Decreased Egg Production in West Java, Indonesia

Nasywa Salsabila[1], Chrysanti[2], and Armanda Dwi Prayugo[2,a,]*

[1]Study Program of Veterinary Medicine, Padjadjaran University, West Java, Indonesia
[2]Department of Biomedical Sciences, Faculty of Medicine, Padjadjaran University, West Java, Indonesia

*Corresponding Author: armanda@unpad.ac.id (Armanda Dwi Prayugo)

ORCID Number: [a] 0000-0002-1627-4917

†These authors contributed equally.

Submitted: 05 Jan. 2026 Revised: 25 Mar. 2026 Accepted: 05 Jun. 2026 Published: 26 Jun. 2026

Abstract

Background: Newcastle Disease (ND) is an endemic in poultry that can cause high mortality, reduced productivity, and significant economic losses in layer farms. A decrease in hen-day egg production may occur without pathognomonic clinical symptoms. Therefore, supportive diagnostic methods are required to detect active virus infection. **Methods**: This study aimed to determine the presence of the Newcastle Disease Virus (NDV), isolated in embryonated chicken eggs (ECE) from organ samples collected from vaccinated layer hens that still decreased the egg production. This study was conducted on four Isa Brown layer hens from two housing at the ABC Farm, West Java, Indonesia. Organ samples included brain, proventriculus, spleen, lung, caecal tonsils and uterus. Hemagglutination (HA) and hemagglutination inhibition (HI) tests were used to identify NDV. **Results**: The NDV was successfully isolated from the brain, proventriculus, spleen, and uterus organ samples using the *in ovo* method and HA test during the first passage with HA titers ranging from 2^5 to 2^7 HAU. HI test confirmed that the detected virus was NDV. **Conclusions**: *In ovo* isolation method combined with HA and HI test can be utilized to detect active NDV infection in layer hens that experience a drop in egg production. The brain, proventriculus, spleen, and uterus are ideal organs to be used as diagnostic samples for ND.

Keywords

Newcastle Disease, *In ovo* virus isolation, Hemagglutination, Layer hens, Drop production

1. Introduction

The poultry sector plays a pivotal role in global food security by providing high-quality protein, essential nutrients, and economic stability, particularly in developing regions. It is one of the fastest-growing agricultural sub-sectors, driven by increasing population, urbanization, and rising incomes, with significant contributions to rural livelihoods and poverty alleviation [1]. In recent years, the layer hen commodities in Indonesia has experienced rapid growth. The layer hen population in Indonesia has reached approximately 414.8 million chickens in 2024 [2]. However, suboptimal egg production continues to be a recurring issue in layer farms, as laying performance may decrease or fail to reach optimal productivity [3]. This situation was recorded at ABC Farm located in Ciparay, Bandung Regency, West Java that currently houses approximately 200.000 chickens. In several housing productive Isa Brown layers aged 23-28 weeks, hen-day

production (HDP) reached only 78%, which is considerably lower than the performance standard of 90-97% at peak production [4]. This condition may be associated with various factors, including infection disease outbreaks such as Newcastle Disease [5].

Newcastle Disease (ND) is a highly contagious poultry disease caused by virulent strains of Avian Avulavirus-1 [6]. The virus is associated with significant mortality rates, which can reach 100% in infections involving velogenic strains [7]. This disease is classified by the World Organization for Animal Health (WOAH) as a notifiable disease and endemic in Indonesia, where various cases are frequently reported [8]. ND significantly impacts layers performance. Research conducted in Subtropical India indicates that production drops in affected farms with ND can reach up to 29% [9]. Furthermore, a study in 2018 observed that in vaccinated layer flocks, ND infection often manifests without high mortality or severe clinical signs, yet still leads to a decline in egg production [10]. A decrease in hen-day egg production may occur without typical clinical symptoms, therefore, reliable diagnostic methods are required to detect active viral infection.

Newcastle Disease can be detected using various diagnostic techniques. However, the gold standard method recommended by the WOAH for identifying the ND virus involves virus isolation in pathogen-free embryonated chicken eggs, followed by characterization using the HA assay [8]. This method enables the detection of live, infectious viruses using organ tissue samples collected via necropsy [11]. The diagnostic value of tissue samples was highlighted by previous research in Assam, India, which demonstrated that ND detection rates were significantly higher in tissue samples (58.78%) compared to cloacal swabs (22.55%) [12]. However, PCR-based methods, particularly reverse transcription polymerase chain reaction (RT-PCR) and its variants, are highly sensitive tools for detecting the presence of NDV by targeting its genetic material. These methods are widely employed in studies investigating NDV detection in organ samples due to their ability to rapidly identify viral RNA with high specificity and sensitivity [13]. Despite their advantages, PCR-based methods do not directly measure active infection, as they detect viral RNA, which may also originate from non-viable or inactivated viruses. This limitation underscores the importance of combining molecular diagnostics with other methods, such as virus isolation, to confirm the presence of infectious virus particles. While PCR methods are more sensitive and faster, they require specialized equipment, skilled personnel, and are more expensive. In contrast, *in ovo* methods are cost-effective and remain the gold standard for assessing viral infectivity, especially for viruses that cannot be propagated in cell culture [14].

Consequently, the application of the gold standard isolation method remains relatively limited, particularly in studies involving layer chickens. This gap is further compounded by the lack of comprehensive data regarding the optimal target organs for diagnostic sampling in layer flocks specifically experiencing declined egg production. Several studies have identified lesions in organs such as the trachea, spleen, and liver. However, most of this research focuses on general pathology and molecular traits rather than organ-specific details on virus isolation [15]. Therefore, this study is crucial to exploring the capability of the *in ovo* method and HA in detecting NDV active infection isolated from the organs of layer chickens in flocks exhibiting decreased egg production from spleen, brain, uterus, proventriculus, caeca tonsil and lungs.

2. Materials and Methods

2.1 Flock History

Cage house M5 housed a population of 8,000 layer hens aged 28 weeks, recording an average egg weight of 58 g and a HDP rate of 78%. The flock's vaccination regimen included the administration of ND and Avian Influenza (AI) vaccines at 12 weeks of age, followed by ND, Infectious Bronchitis (IB), and Egg Drop Syndrome (EDS) vaccines at 14 weeks. The most recent vaccination, consisting of ND and IB, was administered at 22 weeks. Despite this routine immunization program, Cage house M5 experienced a depletion rate of 12% and a mortality rate of 6%.

Similarly, Cage house M6 contained a population of 8,000 hens aged 23 weeks. This flock also recorded a production rate of 78%, with an average egg weight of 53 g. The vaccination schedule mirrored that of Cage house M5: ND and

AI vaccines were administered at 12 weeks, followed by ND, IB, and EDS at 14 weeks. The final vaccination (ND and IB) was given at 21 weeks of age. Consistent with the findings in Cage house M5, Cage house M6 recorded a depletion rate of 12% and a mortality rate of 6%.

2.2 Sample Collection and Extraction

Specimens were collected via necropsy from four layer hens from housing that have decreased egg production. The two birds were collected from each housing (M5 and M6) as samples and euthanasia was carried out using exsanguination method. Targeted organ samples were collected, such as the brain, trachea, lungs, spleen, uterus, and proventriculus. These samples were immediately submerged in activated viral transport medium (VTM) and preserved at 4°C using ice packs during transport, before being stored in a freezer prior to inoculation process in embryonated chicken egg.

One gram of tissue sample was aseptically collected and homogenized using a three way-stopcock and syringe while gradually adding phosphate-buffered saline (PBS) supplemented with 1% gentamycin. After homogenization, the samples underwent three cycles of freeze-thawing. The tissue suspension was then transferred to a sterile microtube and centrifuged at 2.500 rpm for 10 to 15 minutes to remove debris and separate the supernatant. The resulting supernatant was carefully collected and incubated at 37 °C for 30 minutes prior to inoculation into embryonated chicken eggs with Specific Antibody Negative (SAN) specification. For each organ sample, two eggs were used. Following inoculation, the eggs were incubated for 72 hours. Embryos were monitored via candling every six hours; upon detection of death, the eggs were transferred to a chiller overnight prior to the collection of allantoic fluid.

2.3 Virus Inoculation

Inoculation was performed on 9-day-old embryonated chicken eggs. Prior to inoculation, embryonated chicken eggs were candled to confirm viability and to outline the air sac boundary. A small aperture was drilled above the air sac, through which the viral NDV suspension (0,1 mL) with gentamycin 1% was inoculated into the allantoic cavity via a sterile syringe. The puncture site was then sealed with nail polish, and each egg was labeled according to the respective inoculum. The eggs were subsequently incubated at 37 °C and observed at 6 h intervals [16]. The virus was propagated through a series of sequential blind passages. In this process, harvested material was pooled and used to inoculate one egg per step, which served as the source for two consecutive passages (Passage 1 and Passage 2), as detailed in Table 1. Each step egg per organ samples from M5 and M6 cages and one egg as control.

2.4 Allantoic Harvest and Macroscopic Embryo Observation

The harvesting process began by opening the shell above the air sac with forceps and carefully breaching the membranes to avoid vascular damage. Using a sterile microtip, the allantoic fluid was aspirated from an area free of the embryo and yolk sac. The harvested fluid was clarified by centrifugation, and the supernatant was transferred to sterile tubes. Embryos were subsequently examined in petri dishes for pathological changes, and all observations were photographically documented.

2.5 Hemagglutination Assay (HA) and Hemagglutination Inhibition (HI)

The HA was performed in microtiter plates. Twenty five microliters (0.025 mL) of PBS was dispensed into each of the 12 wells. Organ suspension or harvested allantoic fluid was added 0.025 ml to the first wells, followed by two-fold serial dilutions from the second to the eleventh well. Subsequently, 0.025 mL of a 1% red blood cell (RBC) suspension was added to each well. In the HA test, the twelfth well containing PBS and red blood cells served as a negative control. In the HI test, a standardized antigen was added to wells containing serially diluted antiserum before the addition of red blood cells. All samples were tested in duplicate to ensure result validity and consistency. The plate was gently shaken and allowed to stand until hemagglutination reactions occurred. The HA results were observed at room temperature for approximately 15 minutes or until the control wells showed a definitive reaction [16]. Hemagglutination Inhibition test was performed according to WOAH protocols with harvested allantoic fluid from organ samples as antigens. The NDV and AIV subtype H5N1 anti serum was carried out from Centre for Pharmaceutics and

Veteriner Indonesia (PUSVETMA) Surabaya, Indonesia.

2.6 Ethics Statement

All experimental protocols involving samples from animals in this study have undergone a rigorous review process and received formal approval from the Research Ethics Committee of the Faculty of Medicine, Universitas Padjadjaran. This authorization is documented under ethical clearance number 982/UN6.KEP/EC/2025, ensuring full adherence to the established ethical standards for scientific research.

3. Results

3.1 Virus Inoculation

The inoculation process of organ suspensions into ECE was carried out through several stages [11]. Harvesting was performed after embryo death was confirmed or after completion of the incubation period for eggs that did not show mortality, by collecting allantoic fluid and embryonic brain tissue. The result of inoculation stage are presented in Table 1.

The viral inoculation process across successive stages demonstrated increasing adaptation and pathogenicity in ECE (Table 1). During the initial blind passage, only 30.7% (4/13) of the embryos showed mortality within 48 hours. However, upon pooling and first passage, mortality rates increased significantly, with 100% of embryos from both flocks (M5-3 and M6-1) dying within 24–48 hours post-inoculation. This consistent mortality pattern was maintained through Passage 2, where 83.3% (10/12) of embryos succumbed within the same timeframe, confirming the successful isolation and stabilization of the virus in the allantoic fluid.

3.2 Macroscopic Embryo

All harvested embryos were examined and compared with control embryos (Fig. 1A). Several pathological lesions were observed during macroscopic examination. One of the notable pathological findings was embryo dwarfism (Fig. 1B), characterized by a smaller embryo size compared with the control. In addition to dwarfism, other pathological lesions included petechiae (Fig. 1D), congestion (Fig. 1E), and hemorrhage (Fig. 1F), which were observed on all

Table 1. Organ sample inoculation in embryonated chicken eggs (ECE).

Inoculation Stage	Specimen	Result
Blind Passage	Supernatants of extracted organs (brain, lungs, proventriculus, caecal tonsils, spleen, and uterus) from one chicken samples cages M5 and M6 inoculated in to one egg per sample organs.	Four ECE died within 24–48 hours post-inoculation, while nine ECE survived beyond 72 hours
Pooling	Pooled organ supernatants from two layer chickens each in groups M5 and M6 were inoculated into embryonated eggs (one egg per pooled sample).	Seven ECE died within 24–48 hours post-inoculation, one ECE died between 48–72 hours, and four ECE survived beyond 72 hours
Passage 1	Allantoic fluid obtained from inoculated pooled samples of cages M5 and M6 inoculated to one egg per sample.	All ECE from flocks M5 and M6 died within 24–48 hours post-inoculation
Passage 2	Allantoic fluid from first passage inoculated to one egg per sample.	Ten ECE died within 24–48 h post-inoculation, while two ECE survived beyond 72 h

or certain areas of the embryo surface. Ascites were also observed in one embryo sample (Fig. 1C).

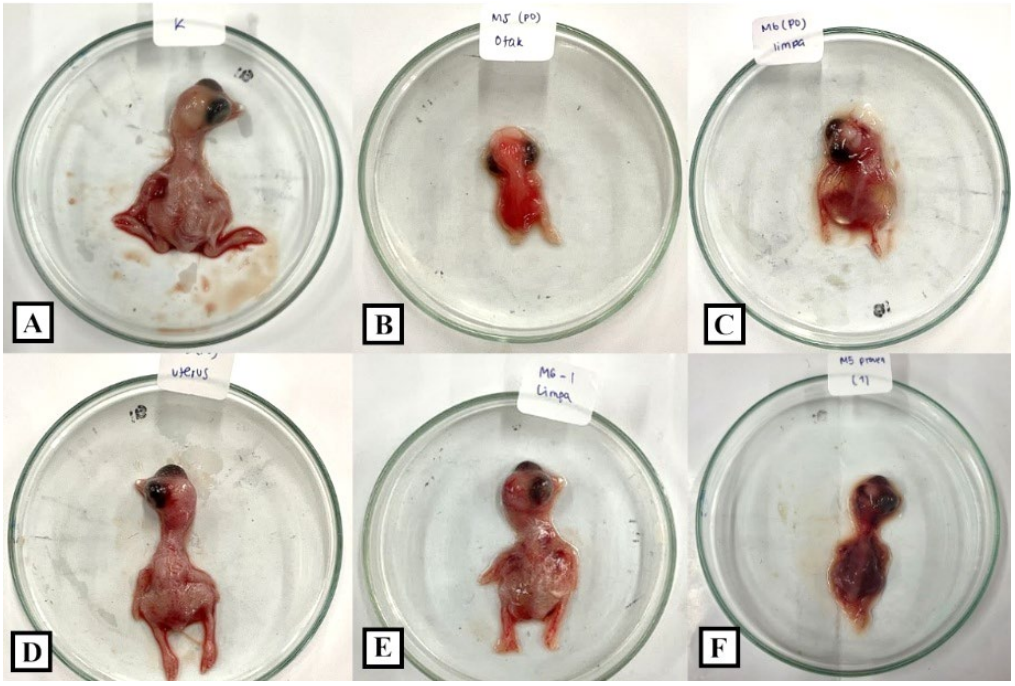

Figure 1. The representative macroscopic lesion of ECE with inoculation from sample organ. A) Normal uninfected chicken embryo (control). B) Embryo dwarfism in inoculated embryo 24 – 36 hours from brain sample. C) Ascites in inoculated embryo 24 – 36 hours from spleen. D) Petechial lesions on the embryo surface death time 48 – 60 hours from uterus. E) Congestion in inoculated embryo death time 48 – 60 hours from spleen. F) Hemorrhage in inoculated embryo observed death time 24 – 36 hours from proventriculus.

Petechial lesions appeared as small red spots distributed in several areas. Congestion was indicated by increased redness of blood vessels, with vessels appearing more prominent beneath the embryonic skin. Hemorrhagic lesions were larger and more extensive than petechiae, presenting as widespread bleeding in all or parts of the embryo. These findings collectively indicate active viral replication following inoculation into embryonated chicken eggs.

The macroscopic findings observed in the ECE during each inoculation stage are summarized in Table 2. In the blind passage, most organ samples from both flocks (M5 and M6) showed no visible lesions, though isolated instances of congestion and hemorrhages in the head or body were noted in specific samples such as M6 proventriculus and M6 uterus. As the process moved to the pooling stage, more distinct pathological changes emerged, including widespread dwarfism and petechial hemorrhages. By Passage 1 and Passage 2, the severity of the lesions progressed significantly; nearly all specimens exhibited extensive hemorrhages over the entire body or concentrated in the head area, accompanied by consistent dwarfism. Notably, by the second passage, several samples resulted in undeveloped embryos, indicating a high level of viral pathogenicity following adaptation.

3.3 Hemagglutination Assay (HA) and Hemagglutination Inhibition (HI)

The results of the HA assay following the blind passage are presented in Table 3. At this stage, viral activity was detected via the partial agglutination in HA test in only one organ sample from Cage house M5, specifically the brain tissue.

In the pooling phase, supernatants originating from the same organs across all

Table 2. Macroscopic findings in embryonated chicken embryos.

Sample	Inoculation Stage			
	Blind Passage	Pooling	Passage 1	Passage 2
M5 Brain	No lesion	Dwarfism	Hemorrhages all over the body, dwarfism	Hemorrhages in head area, dwarfism
M5 Proventriculus	Hemorrhages all over the body	Dwarfism	Hemorrhages in head area, dwarfism	Hemorrhages in head area, dwarfism
M5 Lungs	No lesion	Petechiae all over the body, dwarfism	Hemorrhages all over the body	Hemorrhages in head area, dwarfism
M5 Caecal tonsils	No lesion	Congestion	Hemorrhages all over the body, dwarfism	Undeveloped embryo
M5 Spleen	No lesion	No lesion	Hemorrhages all over the body	Hemorrhages in head area, dwarfism
M5 Uterus	No lesion	Petechiae	Hemorrhages all over the body	Undeveloped embryo
M6 Brain	No lesion	Dwarfism	Hemorrhages in head area	Hemorrhages all over the body, dwarfism
M6 Proventriculus	Congestion	Dwarfism	Hemorrhages all over the body	Undeveloped embryo
M6 Lungs	Hemorrhages all over the body	Petechiae	Hemorrhages in head area	Undeveloped embryo
M6 Caecal tonsils	No lesion	Dwarfism	Hemorrhages all over the body	Undeveloped embryo
M6 Spleen	Congestion	Ascites, dwarfism	Hemorrhages in head area	Congestion, dwarfism
M6 Uterus	Hemorrhages in head area	No lesion	Hemorrhages all over the body	Hemorrhages in head area, dwarfism

Table 3. Hemagglutination assay results from the blind passage samples from cage houses M5 and M6.

	Organ Tissue	HA Test Results[a]	Titer
M5	Brain*	(+)	2^1
M5	Proventriculus	(-)	-
M5	Lungs	(-)	-
M5	Caecal tonsils	(-)	-
M5	Spleen	(-)	-
M5	Uterus	(-)	-
M6	Brain	(-)	-
M6	Proventriculus	(-)	-
M6	Lungs	(-)	-
M6	Caecal tonsils	(-)	-
M6	Spleen	(-)	-
M6	Uterus	(-)	-

[a]HA positive (+) indicates agglutination; negative (-) indicates RBC button.

individual samples within each cage house were combined. The HA results for the pooled brain samples are presented in Table 4.

The results of the HA and HI assays for samples from Cage house M5 are summarized in Table 5. In the first passage, positive HA activity was observed in all organ samples except the lungs, with titers ranging from 2^2 to 2^7. The identity of the isolate was further confirmed through the HI test, which showed positive results for ND but remained negative for AI,

Table 4. Hemagglutination assay results for pooled samples from cage houses M5 and M6.

Code Sample	Organ Tissue	HA Test Results[a]	Titer
M5	Pooled (embryo brain)	(+)	2^5
M6	Pooled (embryo brain)	(+)	2^4

[a]HA positive (+) indicates agglutination.

Table 5. HA and HI assay results from the first and second passage of samples from Cage house M5.

Organ	First Passage				Second passage	
	HA test result[a]	Titer	HI test result (ND)[b]	HI test result (AI)[c]	HA test result[a]	Titer
Brain	(+)	2^6	(+)	(-)	(+)	2^8
Proventriculus	(+)	2^7	(+)	(-)	(+)	2^9
Lungs	(-)	-	(-)	(-)	(-)	-
Caecal tonsils	(+)	2^2	(+)	(-)	(-)	-
Spleen	(+)	2^5	(+)	(-)	(+)	2^{11}
Uterus	(+)	2^7	(+)	(-)	(-)	-

[a]HA positive (+) indicates agglutination; negative (-) indicates RBC button.

[b]HI (ND) positive (+) indicates hemagglutination inhibition (RBC button); negative (-) indicates agglutination.

[c]HI (AI) negative (-) indicates Agglutination.

demonstrating the specificity of the isolated agent. By the second passage, viral titers increased in the brain, proventriculus, and spleen, reaching a peak titer of 2^{11} in the spleen. These findings indicate successful viral propagation and suggest a high affinity of the isolate for various organ tissues, particularly following serial passage.

As detailed in Table 6, the M6 samples demonstrated a clear pattern of viral adaptation where initially negative tissues became positive after further passage. In the first passage, HA activity was only detectable in the proventriculus and uterus. However, by the second passage, both the brain and spleen—which were previously negative—converted to positive with titers of 2^7 and 2^{11}, respectively. This 'negative-to-positive' shift underscores the necessity of serial passage for the recovery of NDV from field samples where the initial viral load may be below the limit of detection.

4. Discussion

4.1 Isolation and Identification of Newcastle Disease Virus

Based on the isolation and identification results, the *in ovo* isolation method was applicable

Table 6. HA and HI assay results from the first passage of samples from Cage houses M6.

Organ	First Passage				Second passage	
	HA test result[a]	Titer	HI test result (ND)[b]	HI test result (AI)[c]	HA test result[a]	Titer
Brain	(-)	-	(-)	(-)	(+)	2^7
Proventriculus	(+)	2^7	(+)	(-)	(-)	-
Lungs	(-)	-	(-)	(-)	(-)	-
Caecal tonsils	(-)	-	(-)	(-)	(-)	-
Spleen	(-)	-	(-)	(-)	(+)	2^{11}
Uterus	(+)	2^8	(+)	(-)	(+)	2^7

[a]HA positive (+) indicates agglutination; negative (-) indicates RBC button.

[b]HI (ND) positive (+) indicates hemagglutination inhibition (RBC button); negative (-) indicates agglutination.

[c]HI (AI) negative (-) indicates Agglutination.

for detecting NDV from organ samples of layer hens in flocks experiencing decreased egg production. In layer flock, decreased egg production is commonly confirmed using additional diagnostic methods such as PCR as one of the available option [13]. However, *in ovo* virus isolation remains the gold standard for confirming live and infectious NDV [8]. A production rate of 78% in Isa Brown layers aged 23 and 28 weeks was considered below the standard performance, which can reach 90–94% under optimal management conditions [9]. The average egg weights in both flocks were also below the standard, at 58 g and 53 g compared with the reference value of 63.1 g [17]. The 6% difference between depletion (12%) and mortality (6%) indicated that a proportion of birds was culled, likely as a preventive measure to control disease spread or due to declining physiological conditions affecting productivity [18].

Newcastle Disease is one of the diseases known to affect egg quality and production in layer hens, particularly velogenic and mesogenic strains. ND infection can result in reduced egg production and reproductive tract lesions [19,20]. Although routine vaccination is effective in preventing severe clinical signs and deterioration of egg quality, it does not fully prevent production decline in infected layer hens [10].

The partial agglutination observed in this organ sample indicates the presence of NDV with hemagglutinating activity in the brain tissue, albeit at a very low level. This finding aligns with previous study, which reported that partial agglutination signifies a genuine interaction between the virus and erythrocytes [21]. Consequently, while the virus is considered biologically present, the extremely low viral load renders the result diagnostically insignificant (below the positive threshold). Despite the low viral load detected by the HA test, macroscopic examination of inoculated embryos revealed pathological lesions, including hemorrhage and congestion. Similar findings have been reported in NDV-infected embryos, where endothelial viral replication leads to vascular damage and widespread hemorrhagic lesions [22,23].

The low viral load observed in organ samples may be attributed to vaccine-induced immune responses that suppress viral replication [10], resulting in predominantly negative findings during the blind passage. Consequently, organ pooling was performed in subsequent stages to improve the sensitivity of virus isolation and detection, as recommended in the WOAH Terrestrial Manual [8]. While pooling samples optimizes surveillance throughput, the resulting HA titer is an indicator of the virus's replicative success in the embryonated egg's allantoic fluid. Samples are pooled to improve the chances of detecting virus in cases where the viral concentration in a single organ may be insufficient for isolation. This strategy ensures that even minimal viral presence is captured and subsequently amplified during incubation in the allantoic cavity [11].

Initial HA testing of allantoic fluid from the pooled organ samples yielded negative results for both Cage houses M5 and M6. However, viral activity was subsequently detected in the pooled embryo brains harvested following the blind passage. The HA assay revealed titers of 2^5 for Cage house M5 and 2^4 for Cage house M6. The result has met the minimum positivity threshold of 2^4, confirming the success of the isolation procedure. The contrast between the positive tissue and the negative allantoic fluid indicates that viral replication occurred in the embryonic tissue but not yet reached detectable levels in the allantoic fluid [24]. Following these results, the allantoic fluid from the pooled samples was passaged into fresh ECE. This first passage was essential to provide an opportunity for extended viral replication from previous allantoic fluid.

The first passage results showed hemagglutinating virus in five of six organ samples from flock M5 and two of six samples from flock M6, indicating successful viral replication. Haemagglutination inhibition testing using specific antisera was conducted to identify the haemagglutinating agent. The isolates showed positive HI reactions with ND antiserum and negative reactions with AI antiserum, confirming that the haemagglutinating virus was NDV and not AI virus, although further serological characterization of other viral antigens is recommended. Based on the second passage results, HA assays confirmed that the ND virus successfully replicated in fresh ECE. Furthermore, examination of the embryos revealed pathological changes consistent with NDV infection.

4.2 Viral Identification in Organ Samples

Based on the isolation and identification results, NDV was detected in several organ samples collected from layer hens showing decreased egg production, with variable viral titers. During the blind passage, virus detected in the brain sample from flock M5 with titers below 2^4 HAU, indicating the presence of virus at very low levels that could not be considered diagnostically positive. The first passage shows that virus was detected in multiple organs from flock M5, including the brain, proventriculus, spleen, cecal tonsils, and uterus, while in flock M6, positive titers were observed in the proventriculus and uterus. The second passage further confirmed virus presence, with increased titers detected in the brain, spleen, and proventriculus of flock M5 and in the brain, spleen, and uterus of flock M6, indicating successful viral amplification.

Utilizing *in ovo* isolation followed by HA and HI tests provides the distinct advantage of detecting biologically active virus from organ samples. This approach is essential for identifying the primary viral drivers of decreased egg production. While embryo mortality within 60 hours post-inoculation [25] serves as a preliminary indicator of velogenic (wild-type) NDV, RT-PCR remains necessary to differentiate between wild-type and vaccine strains. Furthermore, HI testing is critical to rule out Avian Influenza (H5N1) as a confounding cause of production loss. However, a limitation remains: non-hemagglutinating viruses may cause embryo lethality without detection by HA tests. Therefore, a holistic diagnostic approach integrating both serological and molecular assays is required for definitive pathogen characterization.

5. Conclusions

In conclusion, *in ovo* virus isolation combined with HA and HI tests successfully detected NDV in organ samples collected from layer hens experiencing decreased egg production. NDV was predominantly identified in the brain, spleen, proventriculus, and uterus, with varying viral titers after inoculation, indicating that these organs represent the most reliable and optimal samples for ND diagnosis in layer hens with declined egg production, although further serological and molecular characterization is recommended.

Abbreviations

AI, Avian Influenza; ECE, Embryonated Chicken Eggs; EDS, Egg Drop Syndrome; ND, Newcastle Disease; NDV, Newcastle Disease Virus; HA, Hemagglutination Assay; HDP, Hen Day Production; HI, Hemagglutination Inhibition; IB, Infectious Bronchitis.

Availability of Data and Materials

All data are available in this study.

Author Contributions

Conceptualization, A.D.P.; Methodology, N.S., C., and A.D.P.; Investigation, N.S., and A.D.P.; Writing – Original Draft, N.S.; Review & Editing, C., and A.D.P.; Funding Acquisition, A.D.P.; Supervision, C., and A.D.P.

Ethics Approval and Consent to Participate

All experimental protocols involving animals in this study have undergone a rigorous review process and received formal approval from the Research Ethics Committee of the Faculty of Medicine, Universitas Padjadjaran. This authorization is documented under ethical clearance number 982/UN6.KEP/EC/2025, ensuring full adherence to the established ethical standards for scientific research.

Acknowledgment

The authors would like to acknowledge the Microbiology Laboratory C4, Faculty of Medicine, Universitas Padjadjaran, and the Pathology Laboratory, Veterinary Hospital Universitas Padjadjaran for providing laboratory facilities and drh. Fadhli Nanda Putra for technical support during the conduct of this study.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Mottet, A. and Tempio, G. (2017). Global poultry production: Current state and future outlook and challenges. *World's Poultry Science Journal*, *73*(2), 245–256.

[2] Badan Pusat Statistik (2025) *Populasi ayam ras petelur menurut provinsi*, 2024. 1–5.

[3] Sulaiman, D., Irwani, N., and Maghfiroh, K. (2019). Produktivitas ayam petelur strain Isa Brown pada umur 24 – 28 Minggu. *Jurnal Peternakan Terapan*, *1*(1), 26–31.

[4] Anonymous (2022). *ISA brown Management guide*. Hendrix Genetics, Boxmeer: Netherlands.

[5] Ilham, N. and Saptana. (2019). Fluktuasi haga telur ayam ras dan faktor penyebabnya. *Analisis Kebijakan Pertanian*, *17*(1), 27–38.

[6] Emilia, Setiyaningsih, S., and Soejoedono, D. (2015). Isolasi dan karakterisasi biologik virus Newcastle disease. *Jurnal Kedokteran Hewan*, *9*(1), 47–51.

[7] Kerambitan, K., Tabanan, K., Ayu Yuniati Kencana, G., and Gusti Ayu Agung Suartini, I. (2019). Seroprevalensi penyakit tetelo (Newcastle disease) pada ayam buras di. *Indonesia Medicus Veterinus Juli*, *8*(4), 2477–6637.

[8] World Organisation for Animal Health. (2021). Newcastle disease (infection with Newcastle disease virus). In: *Manual of diagnostic tests and vaccines for terrestrial animals, Vol. 2,* 1113-1132.

[9] Sharma, R., Saran, S., Yadav, A.S., Kumar, S., Verma, M.R., Kumar, D., and Tyagi, J.S. (2023). Economic losses due to Newcastle disease in layers in subtropical India. *Indian Journal of Animal Sciences*, *93*(5), 422–426.

[10] Igwe, A.O., Afonso, C.L., Ezema, W.S., Brown, C.C., and Okoye, J.O.A. (2018). Pathology and distribution of velogenic viscerotropic Newcastle disease virus in the reproductive system of vaccinated and unvaccinated laying hens (*Gallus gallus domesticus*) by immunohistochemical labelling. *Journal of Comparative Pathology, 159,* 36-48.

[11] Balachandran, P., Srinivasan, P., Sivaseelan, S., Balasubramaniam, G.A., and Gopala Krishna Murthy, T.R. (2014). Isolation and characterization of Newcastle disease virus from vaccinated commercial layer chicken. *Veterinary World*, *7*(7), 457–462.

[12] Deka, P., Das, S., Deka, P., Nath, M.K., Hazarika, R., Das, G., and Sarma, M. (2022). Molecular detection of Newcastle disease virus from field outbreaks in Assam, India. *The Indian Journal of Veterinary Science and Biotechnology*, *18*(3), 97-99.

[13] Haque, M.H., Hossain, M.T., Islam, M.T., Zinnah, M.A., Khan, R., and Islam, M.A. (2010). Isolation and detection of Newcastle disease virus from field outbreaks in broiler and layer chickens by reverse transcription polymerase chain reaction. *Bangladesh Journal Veterinary Medicine*, *8*(2), 87–92.

[14] Gopinath, V.P., Raj, G.D., Raja, A., Kumanan, K., and Elankumaran, S. (2011). Rapid detection of Newcastle disease virus replication in embryonated chicken eggs using quantitative real time polymerase chain reaction. *Journal of Virological Methods*, *171*(1), 98–101.

[15] Radwan, M.M., Darwish, S.F., El-Sabagh, I.M., El-Sanousi, A.A., and Shalaby, M.A. (2013). Isolation and molecular characterization of Newcastle disease virus genotypes II and VIId in Egypt between 2011 and 2012. *Virus Genes*, *47*(2), 311–316.

[16] Ghadimipour, R., Taghizadeh, M., Bashashati, M., Ebrahimi, M.M., Samadi, A., and Mohammadzadeh, S. (2023). Monitoring of Newcastle disease virus

vaccine strain replication in embryonated chicken eggs by reverse transcription-polymerase chain reaction. *Archives of Razi Institute*, *78*(2), 767–773.

[17] Saleh, K., Pane, D., and Nasution, Z. (2024). Performa ayam petelur isa brown yang diberi ransum LCPD (low crude protein diets). *Jurnal Agri Nauli*, *1(2),*18-27.

[18] Sparrey, J., Sandercock, D.A., Sparks, N.H.C., and Sandilands, V. (2014). Current and novel methods for killing poultry individually on-farm. *World's Poultry Science Journal*, *70*(4), 737–758.

[19] Shofa, M., Wibawan, I.W.T., Zarkasie, K., and Setiyaningsih, S. (2018). Molecular analysis of Newcastle disease virus isolated from a vaccinated layer farm in Indonesia. In: IOP Conference Series: Earth and Environmental Science. 197. 012044 Institute of Physics Publishing: UK.

[20] Bello, M.B., Yusoff, K., Ideris, A., Hair-Bejo, M., Peeters, B.P.H., and Omar, A.R. (2018). Diagnostic and vaccination approaches for Newcastle disease virus in poultry: The current and emerging perspectives. *BioMed Research International*, *Aug 5*(2018), 7278459.

[21] Nieto-Ponce, M., Reyna-Rosas, E., Palencia-Reyes, R.A., Blancas-Ruíz, C., Aguilar-Rafael, G., Rubio-Diaz, M.P.R., Ramirez-Martinez, L.A., Carranza, C., Lozano-Dubernard, B., Torres, M., and Zamudio-Meza, H. (2025). The validation of a hemagglutination inhibition assay that detects antibodies against a Newcastle disease virus-based vaccine vector in human serum samples. *Vaccines*, *13*(4).

[22] Qosimah, D., Murwani, S., Sudjarwo, E., and Arfan Lesmana, M. (2018). Effect of Newcastle disease virus level of infection on embryonic length, embryonic death, and protein profile changes. *Veterinary World*, *11*(9), 1316–1320.

[23] Mariappan, A.K., Munusamy, P., Kumar, D., Latheef, S.K., Singh, S.D., Singh, R., and Dharma, K. (2018). Pathological and molecular investigation of velogenic viscerotropic Newcastle disease outbreak in a vaccinated chicken flocks. *Virus Disease*, *29*(2), 180–191.

[24] Dufour-Zavala, Louise. (Ed.). (2008). *A laboratory manual for the isolation, identification and characterization of avian pathogens* (5th ed.). American Association of Avian Pathologists.

[25] Razzaq, S., Riaz, A., Siddique, N., Saif-ur-Rehman, Shah, M.A., Naeem, K., and Saleem, G. (2025). Evaluation of genotype matched recombinant DNA vaccine for protection against genotype VII velogenic Newcastle disease virus in Pakistan. *Scientific Reports*, *16*, 4402.