UNIVERSITI PUTRA MALAYSIA

PROPAGATION OF LENTOGENIC NEWCASTLE DISEASE VIRUS V4 STRAIN IN SPECIFIC-PATHOGEN-FREE EMBRYONATED CHICKEN EGGS OF DIFFERENT AGES

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PROPAGATION OF LENTOGENIC NEWCASTLE DISEASE VIRUS V4 STRAIN IN SPECIFIC-PATHOGEN-FREE EMBRYONATED CHICKEN EGGS OF DIFFERENT AGES

HAR JING WEI

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Propagation of Lentogenic Newcastle Disease Virus V4 strain in Specific-Pathogen-Free Embryonated Chicken Eggs of Different Ages", by Har Jing Wei and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

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DEDICATION

This thesis is dedicated to my supervisor, Prof Dr. Abdul Rahman Omar, my co-supervisor, Dr. Nor Fitriah Mohamed Sohaimi, senior, Fatin Nursyaza Arman Shah, my family, and friends.

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LIST OF ABBREVIATIONS

NDV	Newcastle disease virus
ND	Newcastle disease
FYP	Final Year Project
SPF	Specific-pathogen-free
ECE	Embryonated chicken eggs
RBCs	Red blood cells
MVP	Malaysian Vaccines and Pharmaceuticals
PBS	Phosphate-buffered saline
AOaV-1	Avian *Orthoavulavirus* serotype-1
D	Day
ELISA	Enzyme linked immunosorbent assay
RT-PCR	Reverse transcriptase polymerase chain reaction
F	Fusion
IFN	Interferons
TLR	Toll-like receptors
IRFs	Interferon regulatory factors
APMVs	Avian Paramyxoviruses
HA	Hemagglutination test
CPE	Cytopathic effects
CAM	Chorioallantoic membrane

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 –Projek Tahun Akhir.

PROPAGASI VIRUS PENYAKIT NEWCASTLE JENIS LENTOGENIK STRAIN V4 DALAM TELUR AYAM EMBRIO BEBAS PATHOGEN TERTENTU YANG BERBAGAI UMUR

Oleh

Har Jing Wei

2023

Penyelia: Prof. Dr. Abdul Rahman Omar

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Penyakit Newcastle (ND) adalah penyakit berjangkit yang sangat menular pada burung yang disebabkan oleh virus penyakit Newcastle (NDV). NDV merupakan virus serotip tunggal yang baru-baru ini dikelompokkan di bawah genus Avian Orthoavulavirus jenis 1 (AOAV-1), dalam keluarga Paramyxoviridae. Virus ini boleh diklasifikasikan lagi sebagai velogenik, mesogenik dan lentogenik berdasarkan patotip virus, di mana pembangunan vaksin hidup dilemahkan adalah berdasarkan strain

NDV lentogenik. Isolasi dan propagasi NDV dalam telur ayam berembrio (ECE) bebas pathogen tertentu (SPF) menggunakan ECE berumur 8-10 hari adalah amalan biasa digunakan dalam mengesan ND dan dalam menghasilkan vaksin. Walau bagaimanapun, ECE yang lebih muda mungkin dapat menyokong pertumbuhan virus disebabkan oleh perkembangan keimunan inat yang kurang lengkap berbanding ECE yang lebih tua. Tambahan pula, ECE yang lebih muda seawal usia sehari telah digunakan untuk menyelamatkan virus genetik berbalik termasuk NDV untuk pembangunan vaksin hidup dilemahkan. Kajian ini adalah sebahagian daripada satu kajian besar dalam menilai umur dan status keimunan ECE dalam menyokong pembiakan vaksin NDV. Oleh itu, kajian ini bertujuan untuk mengkaji perbezaan dalam titer NDV strain V4 lentogenik berikutan inokulasi ECE SPF berumur 3 hari, 5 hari dan 10 hari melalui rongga allantoik dengan dos inokulasi virus pada 10^3, 10^4 dan 10^5. ECE yang diinokulasi dengan larutan saline steril digunakan sebagai kawalan negatif. ECE diperiksa untuk kematian setiap hari dan diinkubasi pada suhu 37C selama 3 hari. ECE yang menunjukkan kematian embrio disimpan pada suhu 4C. Cecair allantoik ECE dikumpulkan untuk mengesan NDV menggunakan ujian Hemagglutination (HA) spot dan ujian HA titration. Keputusan daripada ujian HA titration menunjukkan bahawa titer HA cecair allantoik yang dikumpulkan daripada ECE berumur 10 hari adalah lebih tinggi daripada ECE berumur 5 hari ($P<0.05$). Walau bagaimanapun, penyebaran virus menggunakan ECE berusia 3 hari tidak berjaya kerana tiada cecair alantoik ECE yang diinokulasi menunjukkan keputusan HA yang positif. Terdapat korelasi positif dengan hasil virus apabila virus dengan dos berbeza diinokulasi ke dalam ECE berumur 5 dan 10 hari. Inokulasi virus V4 dos 10^5 ke dalam ECE 5 dan 10 hari menghasilkan titer HA yang tertinggi iaitu 10^5 dan 10^{10}

(P<0.05). Kesimpulannya, ECE berusia 5 hari dan 10 hari boleh menyokong pertumbuhan dan propagasi strain NDV V4 tetapi ECE berusia 10 hari menghasilkan titer virus yang lebih tinggi, ini mungkin menunjukkan bahawa penggunaan ECE berumur 10 hari adalah paling sesuai untuk isolasi, dan propagasi strain NDV lentogenik V4.

Kata kunci: *Virus penyakit Newcastle strain lentogenik V4; umur; telur ayam berembrio; titer*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 - Final Year Project.

PROPAGATION OF LENTOGENIC NEWCASTLE DISEASE VIRUS V4 STRAIN IN SPECIFIC-PATHOGEN-FREE EMBRYONATED CHICKEN EGGS OF DIFFERENT AGES

By

Har Jing Wei

2023

Supervisor: Prof. Dr. Abdul Rahman Omar

Co-Supervisor: Dr. Nor Fitriah Mohamed Sohaimi

Newcastle Disease (ND) is highly contagious viral disease of birds caused by Newcastle disease virus (NDV). NDV is a single serotype virus which recently been grouped under the genus Avian Orthoavulavirus type 1 (AOAV-1) under the family Paramyxoviridae. The virus can be further classified as velogenic, mesogenic and lentogenic based on the virus pathotypes, where the development of live attenuated vaccine is based on lentogenic NDV strains. NDV isolation and propagation in specific-pathogen-free (SPF) embryonated chicken eggs (ECE) are common practices used in diagnosing ND and in producing vaccine using ECE aged 8-10 days. However, younger ECE may be able to support the growth of the virus due to the incomplete development of innate immunity as compared to older ECE. Furthermore, younger ECE as early as one day old has been used to rescue novel reverse genetic viruses including NDV for the develpment of live attenuated vaccine. This study is part of a bigger study in evaluating the ECE age and immune status in supporting the propagation of NDV vaccine strains. Therefore, this study aimed to study the differences in lentogenic NDV V4 strain titers following inoculation of 3-day-old, 5-day-old and 10-day-old SPF ECE via the allantoic cavity with different inoculation doses at 10^3, 10^4, 10^5 of the virus. Sterile saline inoculated ECE of different ages was used as negative controls. The ECE was checked for viability daily for embryonic death. The ECE was incubated at 37C for 3 days. ECE showing embryonic death was chilled at 4C. Allantoic fluid of ECE was harvested to detect NDV based on a hemagglutinin (HA) spot test and HA titration test. Results from the HA titration test showed that the HA titer of allantoic fluid harvested from 10-day-old ECE was significantly higher than 5-day-old ECE ($P<0.05$). However, propagation of the virus using 3-day-old ECE was not successful as none of the allantoic fluid of the inoculated ECE showed positive HA results. There was a positive correlation with virus yield when viruses of different doses were inoculated into 5 and 10-day-old ECE. Inoculation of V4 virus with the dose of 10^5 into 5 and 10-day-old ECE showed the highest mean HA titers of 10^5 and 10^{10}, respectively ($P<0.05$). In conclusion, 5-day-old and 10-day-old ECE can support the growth and propagation of NDV V4 strain

but 10-day-old ECE produces higher virus titer, this may indicate that usage of 10-day-old ECE is most suitable for isolation, and propagation of lentogenic NDV V4 strain.

Keywords: *Lentogenic Newcastle disease virus V4 strain; age; embryonated chicken egg; titer*

1.0 INTRODUCTION

Newcastle Disease (ND) caused by Newcastle disease virus (NDV), is a highly contagious viral disease of birds, including domestic poultry. In poultry, ND can manifest with a wide range of clinical signs, involving the gastrointestinal, respiratory, reproductive and nervous systems, often resulting in mortality rates of up to 100%, depending on the viral pathotype (Miller and Koch, 2013). The initial outbreak of ND was reported on Java Island, Indonesia in 1926, and it subsequently spread globally, including an outbreak in Newcastle-upon-Tyne, England (Doyle, 1927). The disease has since become a global concern, drawing significant attention due to the alarming morbidity and mortality rate and decrease in production and quality of eggs, especially in unvaccinated poultry.

Virus isolation and propagation under controlled laboratory conditions serve as the foundation for diagnosing viral diseases, manufacturing vaccines, and generating recombinant viruses that could potentially be employed in gene therapy. The laboratory diagnosis of ND can be achieved through virus isolation in specific-pathogen-free (SPF) embryonated chicken eggs (ECE), reverse transcriptase polymerase chain reaction (RT-PCR) and detection of antibody based on enzyme-linked immunosorbent assay (ELISA) and virus neutralization test (VNT) (Mao *et al*., 2022). Nevertheless, isolation of NDV in SPF ECE coupled with molecular or serological methods is considered the gold standard for definitive diagnosis of ND (Bello *et al*., 2018). This method has also been extensively utilized for vaccine production due to its convenience (Zhao *et al*., 2012).

The SPF ECE are used for isolation and propagation of various viruses, although the age of the embryo used varies with the different viruses. For NDV inoculation and isolation, ECE aged 8-10 days are typically used (Guy, 2015). The age of the embryo is critical to creating an environment within the eggs that is conducive to virus growth and replication while also allowing for the development of an embryo capable of supporting the viral propagation process.

The immune system of the embryo undergoes continuous development during the 21-day embryonic period, consisting of both innate and adaptive immunity components. Key elements of innate immunity, such as Interferon regulatory factors (IRFs), Toll-like receptors (TLRs) and macrophages, starts their development in chicken embryos as early as day 3 (Hincke *et al*., 2019). Additionally, the study also further suggested that the development of adaptive immunity (B and T cells) occurs on day 11. Hence, the immune competence of ECE at different ages varies can influence the replication and propagation of NDV, resulting in different virus titres. However, various other factors, including nutrients availability, the virulence of the virus and the dose of inoculation may also influence the ability of the ECE to support the virus growth to a high yield (Qosimah *et al*., 2018).

The objective of this study is to investigate the differences in NDV titres obtained from 3-day-old, 5-day-old and 10-day-old SPF ECE inoculated with different dose of the lentogenic NDV V4 strain. The results of this study will contribute to our understanding of whether ECE younger than 10 days old can be a viable option for virus isolation and propagation.

1.1 OBJECTIVES

This study was conducted to determine the viability of SPF ECE of different ages following inoculation with lentogenic NDV strain V4 of different inoculation doses and (2) to determine the presence of NDV from the inoculated SPF ECE based on rapid hemagglutination (HA) spot test and (3) to quantify the NDV titer from the inoculated SPF ECE based on HA titration test.

1.2 HYPOTHESIS

The null hypothesis (H_0) for this study was:

H_0 = There is no significant difference in the NDV titre harvested from 3-day-old, 5-day-old and 10-day-old NDV V4 strain inoculated ECE.

The alternative hypothesis (H_A) was :

H_A = There is a significant difference in the NDV titre harvested from 3-day-old, 5-day-old and 10-day-old NDV V4 strain inoculated ECE.

2.0 LITERATURE REVIEW

2.1 NEWCASTLE DISEASE VIRUS

Newcastle Disease (ND) is caused by the Newcastle disease virus (NDV), which belongs to the species Avian Orthoavulavirus 1(AOAV-1), under the genus Orthoavulavirus and family *Paramyxoviridae* (Rima *et al*., 2019). NDV is characterized by a single-stranded, negative sense, non-segmented enveloped RNA genome. Six essential structural proteins including hemagglutinin-neuraminidase protein (HN), fusion protein (F), matrix protein (M), nucleoprotein (NP), phosphoprotein (P) and large protein (L) are encoded by six genes that make up the genome codes (Ogali *et al.*, 2020). F and HN proteins are two important functional surface glycoproteins of NDV virus. The F protein mediates the fusion of virus and cell membrane. The HN proteins possess antagonistic activities upon interactions with sialic acids-containing receptors on cell surfaces. The H protein attaches to the host cell for initiation of viral infection while the N protein enables the virus to be released from the host cell (Lamb & Kolakodsky, 1996).

ND is endemic throughout Southeast Asia, and it has been considered the most important viral disease of poultry in the region (Alexander, 1997). Currently, ND has been identified in more than 250 avian species, which highlights that virtually all wild and domestic birds are susceptible to the infection. The transmission of ND can occur via direct or indirect contact between infected and healthy birds (Kaleta & Baldauf, 1988). However, NDV is most often transmitted through direct contact with bodily fluids such as urine or oropharyngeal secretions from infected birds. ND can also spread between bird indirectly through contact with contaminated feed or water, farm equipment and vehicles (Jarso, 2015).

The clinical signs and pathology of ND varies depending on the NDV strains and hence none may be considered pathognomonic for ND. ND may cause subclinical disease with no clinical signs to peracute disease that leads to 100% mortality (Ashraf, & Shah, 2014). Hence, NDV is commonly classified into five strains depending on the severity of the disease, namely, viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic enteric (Beard and Hanson, 1984). Both viscerotropic and neurotropic velogenic stains cause high mortality in the infected birds, with viscerotropic strains leading to severe intestinal lesions and neurotropic strains causing respiratory and nervous signs (OIE, 2021). Mesogenic strains show clinical disease but low mortality. Lentogenic strains have low virulence and cause subclinical infection with mild respiratory or enteric diseases (Dortmans *et al.*, 2011).

2.2 LENTOGENIC NEWCASTLE DISEASE VIRUS STRAIN V4

Worldwide, lentogenic live vaccines are the most widely used ND vaccines, as they can induce protective antibody responses and are considered safe at the same time. The lentogenic vaccines are low in virulence and cause acceptable levels of post-vaccinal clinical disease symptoms (Dimitrov *et al*., 2017). This safety profile is vital in maintaining bird health and productivity. Among the lentogenic strains, V4 is among the mildest strain that replicates mainly in the gastrointestinal tract of chickens, while LaSota is far more virulent than V4, which replicates primarily in the respiratory tract of chickens. Nevertheless, both strains have been used for the development and production of live attenuated vaccines. Vaccination with either LaSota or V4 is efficient in protecting the birds against ND, even though the genetic distance between vaccine and challenge virus is significant (Susta *et al*., 2014). They provide sufficient immunity that can prevent the development of clinical signs. LaSota is suitable for those countries where virulent ND is endemic, as this vaccine generally induces high antibody titres (Hu *et al*., 2022). V4 possesses heat-stable hemagglutinin and infectivity, hence, are thermostable vaccines that are widely used in remote areas where preservation of vaccines at low temperature is hard to achieve (Wambura 2006).

2.3 EMBRYONATED CHICKEN EGGS INOCULATION

Embryonated chicken eggs (ECE) were first used for virus isolation and propagation in 1931 by Dr. Goodpasture (Parker, 2004). ECE are readily available, relatively cheap and easily manipulated under sterile conditions. Most avian viruses can be isolated by inoculating the sample into the allantoic cavity, including NDV. The virus replicates in the cells of the allantoic membrane and is shed into the allantoic fluid. Inoculation of NDV virus into the allantoic cavity of ECE is considered the most convenient method to propagate NDV (Mansour et al., 2016). This method is also extensively used to for NDV propagation for research purposes or vaccine production. Ten-day-old embryonated chicken eggs are commonly used for inoculation of NDV (OIE, 2021).

2.4 EMBRYONIC IMMUNE SYSTEM DEVELOPMENT

The immune response in chickens can be subdivided into innate and adaptive immunity. Innate immunity is the body’s first line of defence against microbes and has a non-specific mechanism that responds to all microbes in the same way (Marshall *et al.*, 2018). The major components of the innate immune system in chickens are phagocytic cells and natural killer (NK) cells. The immune system recognises pathogens through the engagement of Toll-like receptors (TLRs) with pathogen-associated molecular patterns (PAMPs). The expression of most TLRs in chick embryonic tissues, such as TLR2A, TLR3, TLR4, TLR5, TLR7, TLR15 and TLR21 starts from ED3 (Kannaki *et al*., 2015). TLRs recognize viral ligands and initiate innate preparedness in the immune system.

Macrophages first appear in the blood circulation and at perivascular regions on the fourth day of embryonic development (ED4), but they are not recruited to incisional wounds (Balic, 2014). Macrophages enter the liver and kidneys on ED12 and ED16, respectively. These macrophages are functionally mature and capable of recognizing and engulfing microbial antigens (Qureshi, 2000). NK cells eliminate virus-infected cells by releasing cytotoxic granules containing granzyme (Prager *et al.*, 2019). NK cells appear in the spleen by ED14, and their number increase by ED19 (Gobel *et al*., 1994).

Adaptive immunity comes into play after innate immunity and specifically targets the type of microbe causing the infection. Adaptive immune comprised of T lymphocytes, B lymphocytes and antibodies. The emigration of T lymphocytes from the thymus to other lymphoid organs and tissues occurs in three waves, on ED6, ED12 and around hatching time, respectively. Additionally, B lymphocyte differentiation begins on ED15, with immature B cells differentiating into plasma cells and memory B cells (Hincke *et al.*, 2018). B cells are first recruited to the secondary lymphoid organs such as the spleen on ED18. Hence, the adaptive immunity of chicken embryos is not effective in producing antibodies (Jankovic *et al.*, 1975). The primary and secondary organs of the immune system, such as the thymus, bursa of Fabricius and cecal tonsils form during embryonic development (Hincke *et al.*, 2018). Peyer's patches and cecal tonsils also start developing on ED13 (Kajiwara, 2003) However, no antibodies are secreted. Mature lymphocytes capable of secreting antibodies are only present approximately 6 days after hatching.

Embryonated eggs obtain the immunoglobulin (Igs) such as IgY (IgG), IgM and IgA from maternal immunity. These Igs are transferred from the hen to the yolk through endocytosis during egg formation. The embryo receives the IgY from the yolk starting from ED7 (Kowalczyk *et al.*, 1985). This selective transport of IgY into the embryo accelerates on ED18 to prepare the immune system against pathogens during and after hatching, pending the B lymphocytes activation and antibody synthesis (Fellah, 2008). Egg white containing IgA and IgM are transferred to amniotic sac on ED12. The embryo received the mixture of egg white and amniotic fluid starting from ED13, allowing IgA and IgM to reach the intestinal mucosa of the embryo, providing enteric protection (Kaspers *et al.*, 1996; Bar-Shira *et al.*, 2014).

3.0 MATERIALS AND METHODS

3.1 VIRUS PREPARATION

The NDV vaccine, lentogenic V4 strain Heat Resistant HR) (Batch No:16304) was obtained from Malaysian Vaccines and Pharmaceuticals Sdn. Bhd. (MVP) and used in this experiment. The vaccine obtained had a titer of $10^{9.5}$/ml and was diluted with ten-fold dilution thrice to prepare the virus with titer of $10^{6.5}$/ml. Then, 0.1ml of virus with titer of $10^{6.5}$/ml was mixed with 0.03ml of PBS to obtain a virus with titer of 10^{6}/ml. The virus with titer of 10^{6} was then diluted with ten-fold dilution thrice to obtain the working inoculation dose which were 10^{5}/0.1ml, 10^{4}/0.1ml and 10^{3}/0.1ml. The virus was filtered with 0.45µm before being injected into the embryonated chicken eggs.

3.2 EXPERIMENTAL DESIGN

Sixty two-day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECE) were obtained from Malaysian Vaccines and Pharmaceuticals Sdn. Bhd. (MVP) and kept in an incubator at 37°C. The ECEs were divided into 12 groups, with each group 5 eggs as follows: Group 1 was Day 3 ECEs inoculated with PBS as negative control, Group 2 was Day 3 ECEs inoculated with 10^{5} /0.1ml of virus, Group 3 was Day 3 ECEs inoculated with 10^{4} /0.1ml of virus, Group 4 was Day 3 ECEs inoculated with 10^{3} /0.1ml of virus, Group 5 was 5-day-old ECEs inoculated with PBS as negative control, Group 6 was 5-day-old ECEs inoculated with 10^{5} /0.1ml of virus, Group 7 was 5-day-old ECEs inoculated with 10^{4} /0.1ml of virus, Group 8 was 5-day-old ECEs inoculated with 10^{3} /0.1ml of virus, Group 9 was 10-day-old ECEs inoculated with PBS as negative control, Group 10 was 10-day-old ECEs inoculated with 10^{5} /0.1ml of virus, Group 11 was 10-day-old ECEs inoculated with 10^{4} /0.1ml of virus and Group 12 was 10-day-old ECEs inoculated with 10^{3}/0.1ml of virus. All the ECEs were monitored daily post-inoculation for 3 days. The viability of eggs was checked every

24 hours. Eggs that died on the first day post-inoculation were discarded. At day 3 post-inoculation, the ECEs were chilled overnight at 4°C. The allantoic fluid of ECEs were harvested to detect the presence of NDV through rapid hemagglutination (HA) spot test. HA titration test was also done to quantify the virus titre of the sample.

3.3 HA SPOT TEST

HA spot test is a direct test that detects agglutination of chicken red blood cells (RBC). Fifty µL of 1% chicken RBC was dispensed onto a white ceramic plate. Fifty µL of allantoic fluid harvested from each egg was added to each drop of blood. The petri dish was gently rotated for one minute to mix the solution well. In allantoic samples containing NDV, there will be agglutination of red blood cells (Figure 1) due to the hemagglutinin-neuraminidase activity of NDV, shown as sandy appearance in the mixture. The absence of a sandy appearance (Figure 2) indicates negative allantoic samples.

Figure 1: Positive HA spot test with sandy appearance

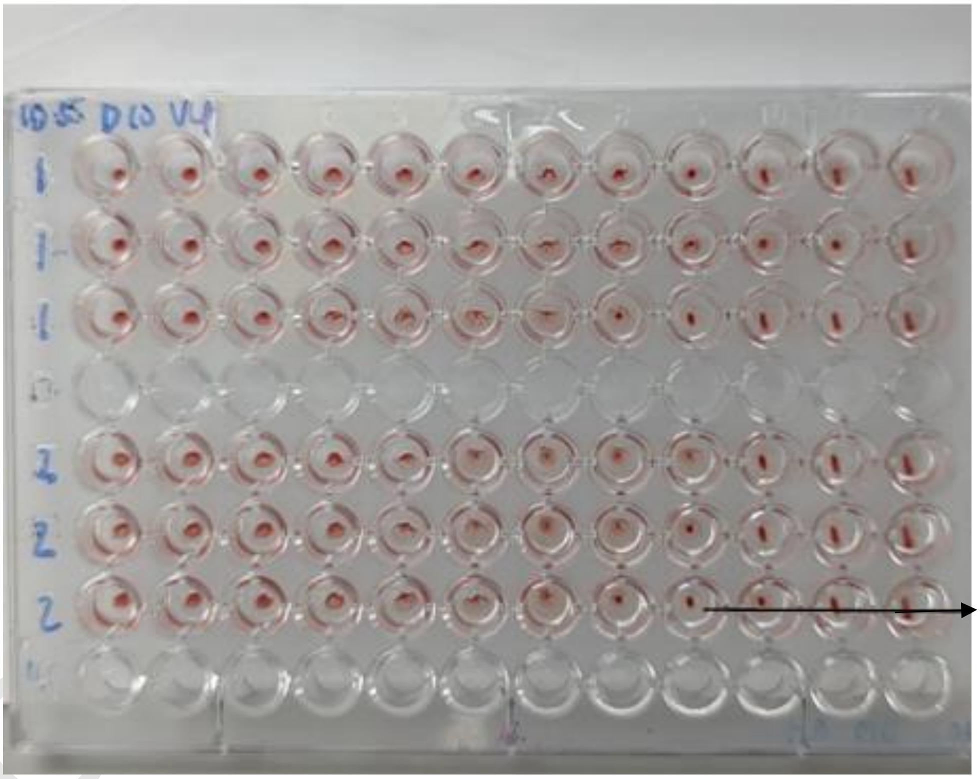
Figure 2: Negative HA spot test with absence of sandy appearance

3.4 HA TITRATION TEST

HA titration test was done to quantify the NDV that causes hemagglutination. 96-well microtitre plates were used for HA titration test. 25 µl of PBS was dispensed into each well. Then, 25 µl of allantoic fluid harvested was added into the first well of each row of column 1. Allantoic fluid from each egg is tested in triplicate. Two-fold serial dilutions of 25 µL of the virus suspension were made across the plate, starting from well no. 1 to well no. 11. Then, 25 µl of 1% chicken red blood cells was placed in each well including column 12. The solution was mixed by tapping the plate gently.

The plate was allowed to stand for 45 minutes at room temperature. Haemagglutination (HA) was determined by tilting the plate and observing for the presence or absence of tear-shaped streaming of the RBCs. The highest dilution showing complete HA (no streaming) was taken as the HA titer. For sample No.2 (Figure 3), the wells at dilution 10^9 show absence of tear-shaped streaming, hence having HA titer of 10^9.

Figure 3: Positive HA titration test showing HA titer of 10^9



Note: The first column of wells contains allantoic samples from 10-day-old ECE inoculated with 10^5/0.1ml of NDV V4 strain. The upper three rows are triplicates of sample No.1, while the lower three rows are triplicates of sample No.2. Two-fold serial dilutions of the virus suspension were made across the plate. The well with highest dilution showing completely absence of tear-shaped streaming was taken as HA titer

3.5 STATISTICAL ANALYSIS

The data collected were arranged into a spreadsheet (Microsoft Excel 365) and reviewed for any missing values or errors. Then, statistical analysis using the IBM® SPSS version 27.0 was done. The HA titer of allantoic fluid harvested from ECEs of different ages were analysed using the Kruskal-Wallis H test. As the confidence interval of this study was 95%, the statistical results were only significant when $P<0.05$.

4.0 RESULTS

4.1 VIABILITY OF SPF ECE POST-INOCULATED WITH NDV V4 STRAIN

The eggs were candled every 24 hours post-inoculation to observe for embryonic death. The viable embryos (Figure 4) moved slowly within the eggs, including slight twitching and rhythmic pulsations. There was also the presence of well-defined blood vessels around the viable embryos. While the dead embryos (Figure 5) were not moving, their blood vessels started to break down and appeared as a streak above the embryo.

Figure 4: Viable Embryos

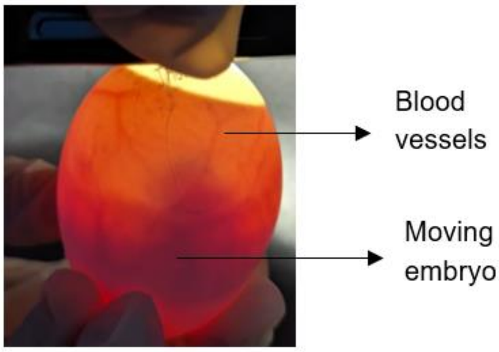
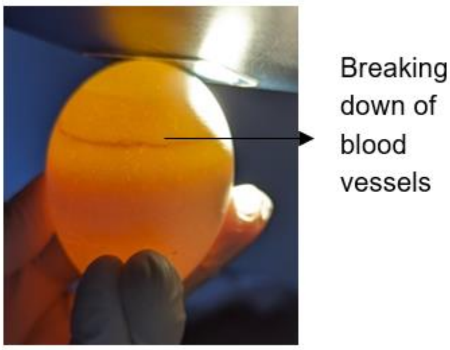


Figure 5: Dead embryos



For the results of 3-day-old ECEs (Table 1), there were four ECEs in dilution 10^3, three ECEs in dilution 10^4 and 10^5 were found alive on day 1 post-inoculation. On day 2 post-inoculation, there were an additional two ECEs of dilution 10^3, one ECEs of dilution 10^4 and two ECE of dilution 10^5 died. All 3-day-old ECE inoculated with NDV were found dead on day 3 post-inoculation.

Table 1: Viability of 3-day-old SPF ECE Post-Inoculation

After inoculation	Inoculation dose	Day 1					Day 2					Day 3				
Viability of ECE	10^3	D	A	A	A	A	D	A	D	A	D	D	D	D	D	D
	10^4	A	D	A	D	D	D	D	A	D	D	D	D	D	D	D
	10^5	A	D	D	A	D	D	D	D	D	D	D	D	D	D	D
	Control	A	A	A	A	A	D	A	D	A	D	D	A	D	D	D

Note: Alive (A), Dead (D)

For the results of 5-day-old ECE (Table 2), there were four ECE in dilution 10^3, three ECE in dilution 10^4 and four ECE in 10^5 were alive on day 1 post-inoculation. However, all 5-day-old ECE inoculated with NDV were found dead on day 2 post-inoculation.

Table 2: Viability of 5-day-old SPF ECE Post-Inoculation

After inoculation	Inoculation dose	Day 1					Day 2					Day 3				
Viability of ECE	10^3	D	A	A	A	A	D	D	D	D	D	D	D	D	D	D
	10^4	D	A	A	A	D	D	D	D	D	D	D	D	D	D	D
	10^5	A	A	A	D	A	D	D	D	D	D	D	D	D	D	D
	Control	A	A	A	D	D	D	A	A	D	D	D	A	A	D	D

Note: Alive (A), Dead (D)

For the results of 10-day-old ECE (Table 3), all five ECE in dilution 10^3 and four ECE in dilution 10^4 and 10^5 were found alive on day 1 post-inoculation, which moved slowly within the eggs when candled. On day 2 post-inoculation, an additional one ECE in dilution 10^3 died with the presence of ruptured vessels. On day 3 post-inoculation, an additional one ECE in dilution 10^5 died.

Table 3: Viability of 10-day-old SPF ECE Post-Inoculation

After Inoculation	Inoculation dose	Day 1					Day 2					Day 3				
Viability of ECE	10^3	A	A	A	A	A	A	A	A	A	D	A	A	A	A	D
	10^4	D	A	A	A	A	D	A	A	A	A	D	A	A	A	A
	10^5	A	A	A	A	D	D	A	A	A	A	D	A	A	A	D
	Control	A	A	A	A	D	A	A	A	A	D	A	A	A	A	D

Note: Alive (A), Dead (D)

4.2 HA SPOT TEST RESULTS

The allantoic fluids of ECE from all three dilutions and control group were harvested and HA spot test was done to determine the presence of NDV in the inoculated 3-day-old ECE. For the 3-day-old ECEs, the allantoic fluids harvested were cloudy, yellowish, and thick in consistency. All the allantoic samples including the control group showed negative results for the HA spot test (Table 4), as there was absence of sandy appearance in the mixture (Figure 6), indicating there was no agglutination of chicken red blood cells (RBCs) due to the absence of NDV.

Figure 6: Negative HA spot test for 3-day-old ECE with absence of sandy appearance

For the results of 5-day-old ECEs, the allantoic fluids harvested were cloudy, yellowish, and slightly thicker as compared to allantoic fluid harvested from 10-day-old embryos. There were four out of five ECEs in dilution 10^3, three out of five ECE in dilution 10^4 and four out of five ECEs in dilution 10^5 showed positive result (Table 5), with sandy appearance in the mixture when observed closely (Figure 7).

Figure 7: Positive HA spot test for 5-day-old ECE with presence of sandy appearance

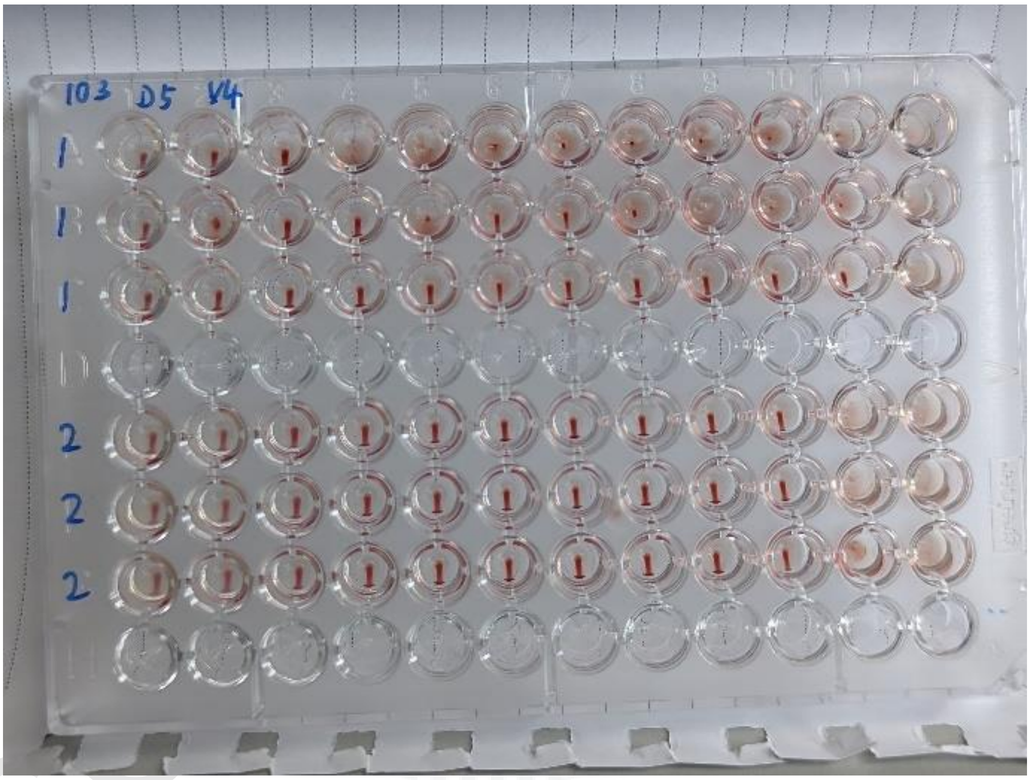
For the results of 10-day-old ECEs, the allantoic fluids harvested were clear and watery in consistency. There were four out of five ECEs in dilution 10^3, 10^4 and 10^5 showed positive results (Table 6), with presence of obvious sandy appearance in the mixture (Figure 8).

Figure 8: Positive HA spot test for 10-day-old ECE with presence of sandy appearance

4.3 HA TITRATION TEST RESULTS

For the 3-day-old embryos, all the allantoic samples showed negative results (Table 4), with all the wells exhibited the presence of tear-shaped streaming of the RBCs (Figure 9).

Figure 9: Allantoic samples from 3-day-old embryos showing negative results for HA titration test



Note: The first column of wells contains allantoic samples from 5-day-old ECE inoculated with 10^3/0.1ml of NDV V4 strain. The upper three rows are triplicates of sample No.1, while the lower three rows are triplicates of sample No.2. Two-fold serial dilutions of the virus suspension were made across the plate. All the wells showed the presence of tear-shaped streaming indicating a negative HA titer result.

For the 5-day-old embryos, only those ECE that showed positive results for HA spot test exhibited HA titers in the HA titration test (Table 5). Among these, the four ECEs in dilution 10^3 displayed HA titers ranging between 10^2 and 10^3 while the three ECEs in dilution 10^4 showed HA titers of 10^4,10^3 and 10^3. The four ECEs in dilution 10 demonstrate HA titers between 10^4 and 10^5.

Similarly, only 10-day-old embryos that tested positive for the HA spot tests exhibited HA titers in the HA titration test (Table 6). Among these, the four ECEs in dilution 10^3 showed HA titers ranging between 10^6 and 10^8 whereas the four ECEs in dilution 10^4 showed HA titers ranging between 10^8 and 10^9. The four ECEs in dilution 10^5 demonstrate HA titers between 10^9 and 10^{10}.

The difference in HA titers of allantoic fluid harvested from 5-day-old and 10-day-old ECE is statistically significant ($P<0.05$). The HA titers of allantoic fluid from 10-day-old ECE were consistently higher than those from 5-day-old ECEs across all three inoculation doses. The HA titers of allantoic fluid from 5-day-old ECE ranged between 10^2 and 10^5 while those from 10-day-old ECE ranged between 10^6 and 10^{10}.

Table 4: Results of HA spot test and HA titration test of 3-day-old ECE

HA spot test							HA titration test					
Inoculation dose	Result					Inoculation dose	Result					Mean HA titer
	No. of eggs						No. of eggs					
	1	2	3	4	5		1	2	3	4	5	
10^5	-	-	-	-	-	10^5	10^3	-	-	-	-	-
10^4	-	-	-	-	-	10^4	10^4	-	-	-	-	-
10^3	-	-	-	-	-	10^3	10^5	-	-	-	-	-
Control	-	-	-	-	-		Control					

Note: Positive (+), Negative (-)

Table 5: Results of HA spot test and HA titration test of 5-day-old SPF ECE

HA spot test							HA titration test					
Inoculation dose	Result					Inoculation dose	Result					Mean HA titer
	No. of eggs						No. of eggs					
	1	2	3	4	5		1	2	3	4	5	
10^5	+	+	+	-	+	10^5	10^4	10^5	10^5	-	10^5	10^5
10^4	-	+	+	+	-	10^4	-	10^4	10^3	10^3	-	10^3
10^3	-	+	+	+	+	10^3	-	10^2	10^3	10^2	10^2	10^2
Control	-	-	-	-	-	Control						

Note: Positive (+), Negative (-)

Table 6: Results of HA spot test and HA titration test of 10-day-old SPF ECE

HA spot test							HA titration test					
Inoculation dose	Result					Inoculation dose	Result					Mean HA titer
	No. of eggs						No. of eggs					
	1	2	3	4	5		1	2	3	4	5	
10^5	+	+	+	+	-	10^5	10^9	10^9	10^{10}	10^{10}	-	10^{10}
10^4	-	+	+	+	+	10^4	-	10^9	10^8	10^9	10^8	10^9
10^3	-	+	+	+	+	10^3	-	10^8	10^6	10^6	10^6	10^7
Control	-	-	-	-	-	Control						

Note: Positive (+), Negative (-)

5.0 DISCUSSION

Embryonated chicken eggs (ECE) have been used for decades as a convenient medium for the virus propagation. However, there is limited research evaluating the use of ECEs younger than 10 days for virus cultivation. The entire chicken embryonic development period spans approximately 21 days, with the immune system developing throughout this timeframe. This is supported by the study by Balic *et al.* (2014), which reported the first appearance of macrophages engulfing pathogens in the blood circulation when embryos reach 5 days old. Additionally, Hincke *et al.* (2019) also stated that T cells and B cells commence development in 10-day-old ECE. Hence, the lentogenic Newcastle disease virus (NDV) V4 strain may replicate more efficiently in ECEs younger than 10 days due to their incomplete immune system development as compared to older ECE.

In the present study, HA spot test was conducted to detect the presence of NDV in allantoic fluid of ECE post-inoculation with NDV. The results indicated the presence of NDV in both 5-day-old and 10-day-old ECEs, inoculated with three different doses. However, a significant difference was observed between the HA titer of allantoic fluid harvested from 5-day-old and 10-day-old ECE. The HA titer, representing the amount of virus needed to agglutinate an equal volume of a 1% chicken RBC suspension, was determined through HA titration test. The allantoic fluids obtained from 10-day-old NDV inoculated ECE showed higher HA titers compared to those from 5-day-old NDV inoculated ECE, across all three different inoculation doses (10^3, 10^4, 10^5). This suggests a notable age-dependent variation in the response of ECE to NDV, as reflected in the observed differences in HA titers.

On the other hand, the allantoic fluid harvested from 3-day-old NDV inoculated ECES yielded negative results in both the HA spot test and HA titration test. This inconsistency suggests an unsuccessful propagation of virus in 3-day-old ECEs. Several factors may contribute to this inconsistency and the unsuccessful inoculation of the NDV V4 strain. The enterotropic nature of the NDV strain, V4, primarily involves replication in the gastrointestinal tract (Cvetić *et al.*, 2021). The V4 virus, upon inoculation, typically replicates initially at the allantoic membrane and then spreads to infect the intestinal mucosa. However, in the case of 3-day-old ECEs, the V4 virus is unable to propagate in the underdeveloped intestinal mucosa of 3-day-old ECES. As highlighted by Huycke and Tabin (2018), the first layer of circular smooth muscle of small intestines begins differentiation when embryo reaches 6 days old, causing structural development of the intestinal epithelium and the underlying mesenchyme. Consequently, the 3-day-old NDV may not be a viable option for the isolation and propagation of NDV due to the lack of suitable conditions for viral replication.

The mortality rates for younger embryos (5-day-old and 3-day-old) NDV inoculated ECEs were consistently higher than that for the 10-day-old embryos, at both day 2 and day 3 post-inoculation. This result is consistent with the previous study done by Jacobsen (2010), who reported that younger ECEs infected with Aspergillus fumigatus showed higher mortality rates. Younger embryos possess less developed tissues and organs compared to 10-day-old embryos. The maturity of embryonic organs contributes significantly to the overall resilience and the ability of chicken embryos to cope with stress factors, including viral infections. On top of that, the immune system of younger embryos, especially the innate immunity that plays a vital role in limiting viral infection in the early embryonic period is also less developed. Although innate immunity starts developing during embryogenesis, it becomes more robust as the embryo matures. According to Sekellick *et al.* (1990), to develop interferon (IFN) systems, cells from younger embryos need more time as compared to those from older embryos. Hence, younger embryos, which have both less developed organs and immune systems are more susceptible to early death due to viral infection.

The allantoic fluids from NDV inoculated ECEs were harvested at day 3 post-inoculation to achieve the highest virus yield. Following the guidelines form the Malaysian Vaccines and Pharmaceuticals Sdn. Bhd. (MVP), the ideal timeframe to harvest the virus is between 68 to 72 hours post-inoculation to obtain high virus titer. However, in the case of 5-day-old ECEs, all embryos were found dead within 48 hours post inoculation. The premature mortality observed in these embryos indicated that the virus may not had sufficient time to replicate to its maximum capacity. Since viruses require living cells for survival and replication, the rapid death of cells in 5-day-old ECEs may have hindered the full replication potential of the virus. This limitation in the availability of viable cells could be a significant factor contributing to the observed lower HA titers in the allantoic fluid harvested from 5-day-old ECEs.

6.0 CONCLUSION

In conclusion, the null hypothesis for this study is rejected, as there is significance difference in NDV titer harvested from 5-day-old and 10-day-old ECE inoculated with the NDV V4 strain. Furthermore, our findings indicate that 10-day-old ECE, when inoculated with NDV V4 strain of three different doses (10^3, 10^4, 10^5), exhibit lower mortality rates and higher NDV titers compared to their 5-day-old ECE counterparts. Hence, 10-day-old ECE emerges as the preferred choice for NDV V4 strain inoculation. Importantly, both 5-day-old and 10-day-old ECE proved capable of supporting NDV V4 strain propagation, while 3-day-old ECE did not demonstrate this capability.

7.0 RECOMMENDATION

The recommendations for future studies include the use of real-time PCR and investigating immune-related genes study to gain a thorough understanding of the immune response in ECE following inoculation with the NDV V4 strain. It is essential to explore the potential correlation between the expression profile of innate immunity genes and the observed differences in virus titer among embryos of different ages. This will contribute to a more comprehensive understanding of the host-virus interaction dynamics.

Additionally, attention should be directed towards enhancing the handling protocols for ECE. The notable occurrence of embryo deaths within 24 hours post-inoculation, primarily attributed to non-specific causes such as bacterial contamination or injury during inoculation (James, 2015). Hence, handling of these fragile early age embryo requires more efficient and refined protocols to minimize stressors and potential sources of contamination, thereby ensuring the reliability and integrity of experimental results.

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