UNIVERSITI PUTRA MALAYSIA

EVALUATING THE ABILITY OF SPECIFIC-PATHOGEN-FREE EMBRYONATED CHICKEN EGGS OF DIFFERENT AGE TO SUPPORT THE PROPAGATION OF LENTOGENIC NEWCASTLE DISEASE VIRUS STRAIN LASOTA

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FACULTY OF VETERINARY MEDICINE

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CERTIFICATION

It is hereby certified that we have read this project paper entitled “Evaluating the ability of specific-pathogen-free embryonated chicken eggs of different age to support the propagation of lentogenic Newcastle disease virus strain LaSota.” by Karen Ooi Feng Shinn and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement of the course VPD 4999-Project.

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DEDICATION

This thesis is dedicated to my supervisor, Prof. Dr. Abdul Rahman Omar, my co-supervisor, Assoc. Prof. Dr. Nor Yasmin Abd Rahaman, senior, Fatin Nursyaza Arman Shah, my family, and friends.

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CONTENTS

TITLE.....i

CERTIFICATION.....ii

DEDICATION.....iii

ACKNOWLEDGEMENTS.....iv

CONTENTS.....v

LIST OF ABBREVIATIONS.....vii

LIST OF FIGURES.....viii

LIST OF TABLES.....ix

ABSTRAK.....x

ABSTRACT.....xiii

1.0 INTRODUCTION.....1

1.1 OBJECTIVE.....2

1.2 HYPOTHESIS.....3

2.0 LITERATURE REVIEW.....3

2.1 NEWCASTLE DISEASE VIRUS.....3

2.2 SOURCE AND MODE OF TRANSMISSION.....4

2.3 CLINICAL SIGNS.....4

2.4 DIAGNOSIS.....5

2.5 PREVENTION AND CONTROL.....7

2.6 DEVELOPMENT OF IMMUNE SYSTEM OF EMBRYONATED CHICKEN EGG (ECE).....10

3.0 MATERIALS AND METHODS

@COPYRIGHT UPM

3.1 VIRUS 12
3.2 SPECIFIC-PATHOGEN FREE EMBRYONATED CHICKEN EGG 12
3.3 VIRUS PREPARATION 13
3.4 VIRUS INOCULATION 14
3.5 HARVESTING ALLANTOIC FLUID OF SPF ECE 15
3.6 HEMAGGLUTINATION SPOT TEST 15
3.7 HEMAGGLUTINATION HITRATION TEST 16
3.8 STATISTICAL ANALYSIS OF HEMAGGLUTINATION (HA) TITER OF 3-DAY OLD, 5-DAY OLD AND 10-DAY OLD ECE 17
4.0 RESULTS 18
4.1 EMBRYONIC VIABILITY OF DAY 3, 5 AND 10 SPF ECE FOLLOWING NDV STRAIN LASOTA INOCULATION 18
4.2 HA SPOT TEST OF 3-DAY OLD SPF ECE 20
4.3 HA TITRATION TEST OF 3-DAY OLD SPF ECE 20
4.4 HA SPOT TEST OF 5-DAY OLD SPF ECE 23
4.5 HA TITRATION TEST OF 5-DAY OLD SPF ECE 23
4.6 HA SPOT TEST OF 10-DAY OLD SPF ECE 26
4.7 HA TITRATION TEST OF 10-DAY OLD SPF ECE 26
4.8 STATISTICAL ANALYSIS OF HA TITER OF 3-DAY OLD, 5-DAY OLD AND 10-DAY OLD ECE 29
5.0 DISCUSSION 30
6.0 CONCLUSION 33
7.0 LIMITATION AND RECOMMENDATION 33
8.0 REFERENCES 34

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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
HI	Hemagglutination inhibition
CPE	Cytopathic effects
CAM	Chorioallantoic membrane
qPCR	Real-time PCR
dpi	Day post-inoculation

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LIST OF FIGURES

Page

Figure 1: Preparation of serial dilution of NDV LaSota strain …………….. 14

Figure 2: HA spot test of 3-days old ECE…………………………………… 22

Figure 3: HA titration test of 3-days old ECE ……………………………… 22

Figure 4: HA spot test of 5-days old ECE ……………………………………. 25

Figure 5: HA titration test of 5-days old ECE ……………………………… 25

Figure 6: HA spot test of 10-days old ECE ………………………………… 28

Figure 7: HA titration test of 10-days old ECE ………………………………. 28

Figure 8: Example of viable embryos with well-defined blood vessels (black arrow) and moves in response to light (yellow arrow) …………… 31

Figure 9: Example of dead embryos with ruptured vessels (black arrowhead) and dark ring appearance around egg (yellow arrowhead) ………… 31

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LIST OF TABLES

Page

Table 1: Embryonic viability of day 3, 5 and 10 embryonated chicken eggs 18

Table 2: Results of HA spot test and HA titration test of day 3 embryonated chicken eggs 21

Table 3: Results of HA spot test and HA titration test of day 5 embryonated chicken eggs 24

Table 4: Results of HA spot test and HA titration test of day 10 embryonated chicken eggs 27

Table 5: Descriptive statistic............ 29

Table 6: Result of the Shapiro-Wilk test............ 29

Table 7: Kruskal-Wallis test............ 30

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti PerubatanVeterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek Tahun Akhir.

MENILAI KEMAMPUAN TELUR AYAM TEREMBRIONAT BEBAS PATOGEN SPESIFIK BERBEZA UMUR UNTUK MENYOKONG PEMBIAKAN VIRUS STRAIN LASOTA LENTOGENIK PENYAKIT NEWCASTLE

Oleh

Karen Ooi Feng Shinn

2023

Penyelia: Prof. Dr. Abdul Rahman Omar

Penyelia Bersama: Prof. Madya Dr. Nor Yasmin Abd Rahaman

Penyakit Newcastle (ND) adalah penyakit virus ayam yang disebabkan oleh Newcastle Disease Virus (NDV). ND adalah kebimbangan utama di seluruh dunia kerana kewujudannya yang berterusan walaupun terdapat program vaksinasi yang intensif. Terdapat 3 patotip NDV, iaitu velogenik, mesogenik dan lentogenik. Terdapat pelbagai ujian diagnostik untuk mengesan NDV, namun kaedah paling mudah untuk mengesan dan mempropagasi NDV adalah dengan inokulasi rongga allantoik telur ayam berembrio. Banyak kajian menunjukkan penggunaan telur ayam berembrio berumur 9-11 hari untuk propagasi NDV, tetapi terdapat kajian terhad yang

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menunjukkan penggunaan telur ayam berembrio muda untuk menumbuhkan virus kerana mereka mempunyai perkembangan imuniti inat yang tidak lengkap berbanding embrio yang lebih tua. Pertumbuhan NDV dalam embrio muda telah digunakan untuk penyelidikan dan pembangunan dalam menyelamatkan virus ND baru dan kajian genetik songsang. Kajian ini adalah sebahagian daripada kajian yang lebih besar dalam memahami perkembangan sistem imun telur berembrio dalam mempengaruh pertumbuhan virus bagi pembangunan vaksin. Oleh itu, kajian ini bertujuan untuk menentukan keupayaan telur berembrio bebas patogen spesifik dengan umur yang berbeza berikutan inokulasi dengan NDV lentogenik strain LaSota pada dos inokulasi berbeza dan untuk mengesan kehadiran NDV menggunakan ujian spot hemagglutination (HA) dan titrasi HA. Embrio berumur 3, 5 dan 10 hari telah disuntik dengan NDV LaSota melalui cecair allantoik dalam tiga dos yang berbeza; 10^3, 10^4 dan 10^5. Telur berembrio yang diinokulasi steril PBS digunakan sebagai kawalan. Telur tersebut diinkubasi pada inkubator 37C dan kematian embrio diperhatikan dan direkodkan selepas inokulasi selama 3 hari. Cecair alantoik semua telur dituai untuk ujian titik HA dan ujian titrasi HA. Pada hari ke-3 selepas inokulasi, telur berembrio inokulasi berumur 3 dan 5 hari mempunyai mortaliti 15/15 telur (100%) bagi semua dos pencairan manakala telur inokulasi berumur 10 hari mempunyai mortaliti 5/15 telur (33%) dengan 1/ 5 telur kematian bagi dos 10^3, 3/5 telur bagi dos 10^4, dan 1/5 telur bagi dos 10^5. Berdasarkan analisis statistik, titer HA antara telur berumur 3, 5 dan 10 hari adalah berbeza dengan ketara di mana telur berembrio 10 hari mempunyai titer NDV tertinggi diikuti dengan teur berembrio 5 hari dan 3hari. Kesimpulannya, semua telur berembrio tanpa mengira umur dapat menyokong pertumbuhan NDV strain LaSota, tetapi telur berembrio 10 hari mempunyai kadar kematian yang lebih

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rendah (33%) berbanding telur berembrio berumur 3 dan 5 hari dan mempunyai titer NDV tertinggi. Telur berembrio berumur 10 hari adalah umur yang paling sesuai untuk menyokong pertumbuhan NDV strain LaSota. Ekspresi gen berkaitan keimunan inat telur berembrio yang berlainan umur dalam modulasi propagasi NDV LaSota sedang dalam kajian.

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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.

EVALUATING THE ABILITY OF SPECIFIC-PATHOGEN-FREE EMBRYONATED CHICKEN EGGS OF DIFFERENT AGE TO SUPPORT THE PROPAGATION OF LENTOGENIC NEWCASTLE DISEASE VIRUSSTRAIN LASOTA

By

Karen Ooi Feng Shinn

2023

Supervisor: Prof. Dr. Abdul Rahman Omar

Co-Supervisor: Assoc. Prof. Dr. Nor Yasmin Abd Rahaman

Newcastle Disease (ND) is a viral disease of poultry caused by Newcastle Disease Virus (NDV ND is a major worldwide concern due to its persistent existence despite intense vaccination programmes. There are 3 pathotypes of NDV, which are velogenic, mesogenic and lentogenic. Numerous diagnostic tests are available to detect NDV, yet the most convenient method to detect and propagate NDV is by inoculation of allantoic cavity of embryonated chicken eggs (ECE). Many studies indicated the use of 9-11 days of ECE to propagate NDV, but there are limited studies indicating the use of young ECE to grow viruses since they have incomplete development of innate immunity compared to older ECE. Propagation of NDV in young ECE has been used for research and development in rescuing novel and reverse genetic ND viruses. This

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study is part of a bigger study in understanding the development immune system of ECE influencing virus growth for vaccine development. Hence, this study aimed to determine the viability of specific-pathogen-free (SPF) ECE of different ages following inoculation with lentogenic NDV strain LaSota of different inoculation doses and to detect the presence of NDV using rapid hemagglutination (HA) spot test and HA titration test. ECE of 3, 5 and 10 days were inoculated with NDV LaSota strain via allantoic cavity in three different doses; 10^3, 10^4, 10^5. Sterile saline inoculated ECE were used as controls. The ECE were incubated at 37C incubator and embryonic viability was observed via egg candling and recorded daily post-inoculation for 3 days. The allantoic fluid of all ECE was harvested for HA spot test and HA titration test. On day 3 post-inoculation, inoculated ECE of 3 and 5-days old had 15/15 ECE (100%) mortality in all dilution doses while 10-days old inoculated ECE had 5/15 ECE (33%) mortality with 1/5 ECE mortality in dose 10^3, 3/5 ECE in dose 10^4, and 1/5 ECE in dose 10^5. Based on statistical analysis, HA titer between 3, 5 and 10-days old ECE are significantly different where day 10 ECE had the highest NDV titer followed by day 5, and day 3 ECE. In conclusion, all ECE of irrespective age are able to support propagation of NDV LaSota strain but day 10 ECE had a lower mortality rate (33%) compared to 3 and 5-days old ECE and had the highest NDV titer. ECE of 10-days old is the most suitable age to support the propagation of NDV LaSota strain. The expression of innate immunity related genes of ECE of different age in modulating the propagation of NDV LaSota is currently under study.

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1.0 INTRODUCTION

Newcastle Disease (ND) is a viral disease of poultry that is caused by Newcastle Disease Virus (NDV) which is classified as Avian Orthoavulavirus-1 (AOAV-1), previously known as Avian Paramyxovirus Type-1 (APMV-1). ND is often a fatal disease in poorly vaccinated chickens, hence it is a major worldwide concern (Ashraf and Shah, 2014). NDV strains have been categorized into three groups: velogenic (virulent), mesogenic (moderately virulent) and lentogenic (mild virulent). Presently, mesogenic and lentogenic NDV have been used as vaccines to control and prevent ND in poultry (Hu *et.al.*, 2022). This study will use a lentogenic LaSota strain, a pneumotropic live attenuated vaccine strain that replicates primarily in the respiratory tracts of chickens.

ND can be diagnosed using laboratory techniques such as virus isolation, reverse-transcriptase polymerase chain (RT-PCR), enzyme-linked immunosorbent assay (ELISA) and sequencing of virus fusion (F) gene However, the most convenient method of propagating NDV is by inoculation of allantoic cavity of embryonated chicken eggs (ECE) (Mansour *et al.*, 2016). ECE is an ideal substrate for viral growth and replication, sterile and free from contaminating bacteria and many latent viruses. In addition, viral inoculation into ECE is also cost-effective, less labor and maintenance needed. Besides, 9 to 11 days of specific-pathogenic-free (SPF) ECE are widely used to grow NDV for vaccine production.

There are two main types of immune defense in chickens which are innate immunity which acts on pathogens in a non-targeted manner and adaptive immunity which targets specific pathogens. Innate immunity comprises interferons (IFNs), Toll-like receptors (TLRs) and phagocytosis, all with antimicrobial properties that

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can directly control the replication or spread of pathogens (Hincke *et al.*, 2019). Embryonic development takes 21 days and usually the first signs of a developing immune system are at ECE 10. However, interferon regulatory factors (IRFs) and TLRs that recognize and respond to viral ligands are expressed from ECE 3 onwards. By ECE 4 or ECE 5, embryonic macrophages can be found in the blood vessels and perivascular region, they can recognize and phagocytose microbial antigens. By ECE 18, the chicken embryo is immunocompetent and capable of eliciting both innate and adaptive responses to pathogens (Hincke *et al.,* 2019). Hence, a young ECE should readily support NDV replication since the embryo's immune components are not fully developed compared to a 9 to 11 days-old ECE provided all the other nutrients and factors required for virus growth are present (Santhakumar *et.al.*, 2018). In addition, reverse genetic studies used young SPF ECE to rescue genetically engineered viruses (Cardenas and Afonso, 2017). From Cardenas's study, as early as 1-day old ECE are used to help propagate the rescued virus in reverse genetic experiment of NDV.

Thus, this study aims to study the differences in NDV titer from 3-day-old, 5-day-old and 10-day-old specific-pathogen-free (SPF) embryonated eggs inoculated with lentogenic NDV strain LaSota of different inoculation doses.

1.1 OBJECTIVE

The objectives of this study are

(1) to determine the viability of SPF ECE of different ages following inoculation with lentogenic NDV strain LaSota of different inoculation dose

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(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 is the amount of NDV harvested from Day 3 ECE, Day 5 ECE and Day 10 ECE are not significantly different. The alternative hypothesis (H_A) for this study is the amount of NDV harvested from Day 3 ECE, Day 5 ECE and Day 10 ECE are significantly different.

2.0 LITERATURE REVIEW

2.1 NEWCASTLE DISEASE VIRUS (NDV)

Newcastle disease (ND) is one of the most important infectious viral diseases of poultry. It is distributed worldwide and potentially causes large economic losses in the poultry industry. (Lancaster, 1976). The causative agent of ND is Newcastle Disease Virus (NDV) which is a paramyxovirus. NDV are enveloped, non-segmented, negative-sense RNA viruses, which - together with the Pneumovirinae - constitute the family of Paramyxoviridae (Mayo, 2002).

Based on pathogenic studies, ND is categorized into three groups: lentogenic (low virulence), mesogenic (moderate virulence) and velogenic (highly virulent). Velogenic ND may result in 100% mortality in poultry leading to significant impact on trade restrictions and embargoes in the regions of its outbreak (Ganar *et al.*, 2014). Meanwhile, mesogenic NDV strains are of intermediate virulence causing respiratory

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infection with moderate mortality (< 10%). Lastly, lentogenic NDV strains cause subclinical infection with mild respiratory or enteric disease and are considered low-virulent (Dortmans *et al.*, 2011). Presently, mesogenic and lentogenic NDV have been used as vaccines to control and prevent ND in poultry (Hu *et.al.*, 2022).

2.2 SOURCE AND MODES OF TRANSMISSION

First and foremost, infected poultry including wild birds may be the sources of NDV. NDV can be transmitted to susceptible birds via direct or indirect contact with infected birds, most often by direct contact with diseased or carrier birds (OIE, 2021). Examples of direct contact included direct contact with feces and respiratory discharges or by contaminated food, water, equipment, and human clothing. Generally, NDV can survive in the environment for weeks, especially in cool weather (OIE, 2021). Besides that, there are strong evidence that wild avian species have been implicated in the transferring of NDV to domestic chicken and wild bird populations as biological carriers or natural reservoirs of NDV (Kim *et.al*, 2007). In short, ND is very contagious and once it is introduced into a susceptible flock, the susceptible flocks will be infected within 2-6 days (OIE, 2021).

2.3 CLINICAL SIGNS

Clinical signs can be one of the diagnostic methods of NDV, but clinical signs alone do not provide or support a reliable basis for ND diagnosis, as the clinical signs vary widely and are dependent on factors such as: the strain of the virus, the species of bird infected, the age of the host, (young birds are the most susceptible), concurrent infection with other organisms, environmental stress and immune status (OIE, 2021).

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Apart from that, the clinical signs shown vary depending on whether the infecting virus has tropism on the respiratory, digestive, or nervous systems (Aiello and Moses, 2016).

There are 3 pathotypes of NDV, velogenic pathotype which is the most virulent can cause severe and sudden death that may reach almost 100% mortality in unvaccinated chicken, chickens may have dyspnea, cyanosis, nervous signs such as tremors, spams, circling and complete paralysis etc. Vaccinated birds may not show any signs except for the decrease in egg production. Mesogenic pathotype which is moderately virulent may cause an acute respiratory disease with nervous signs, decrease in egg production and lower mortality (<10%) compared to velogenic pathotype. Lastly, lentogenic pathotype which is low virulent can cause subclinical disease such as mild respiratory signs and minimal mortality. Examples of respiratory signs include gasping, coughing, sneezing and rales. Mortality is insignificant in lentogenic form. Besides, lentogenic strains are usually used in vaccine production (OIE, 2021).

2.4 DIAGNOSIS

The clinical signs and gross lesions due to infection with virulent Newcastle disease virus (NDV) are not pathognomonic and therefore cannot be used alone for diagnosis of Newcastle disease. Clinical signs and gross lesions can aid in diagnosis of ND but laboratory confirmation plays a crucial role in diagnosis of ND (Kiril, 2023). Furthermore, poultry respiratory pathogens such as avian influenza, infectious bronchitis, and infectious laryngotracheitis viruses are all considered differential

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diagnoses that can easily be confused with NDV based on their clinical presentation (Piacenti *et.al.*, 2006).

There are several diagnostic methods, firstly virus isolation where NDV can be isolated from oropharyngeal or cloacal swabs or tissues from infected birds by inoculation of the allantoic cavity of 9- to 11-day-old SPF embryonated chicken eggs (Kiril, 2023). Specifically, the choice of samples required for virus isolation is determined by the sites of virus replication and routes of viral shedding. In live birds, samples required include the cloacal and oropharyngeal swabs collected in isotonic solution with or without antibiotics. If the birds are already moribund or have recently died, samples should include lungs, kidney, liver, intestine, spleen, and cecal tonsils collected separately or as a pool, in addition to the cloacal and oronasal swabs. Next, to isolate NDV, processed samples are primarily inoculated into the allantoic cavity of 9-10-day-old specific antibody free chicken embryonated eggs. After about 4-7 days of incubation, hemagglutination test (HA) is used to detect the presence of the virus in the infected allantoic fluid. However, since other viruses such as avian influenza and Avian Paramyxoviruses (APMVs) might also possess HA activity, it is always necessary to further confirm the identity of the virus using other diagnostic tests such as hemagglutination inhibition test (HI) using NDV specific antisera or molecular tests. The simplest and most inexpensive serological test for NDV is HI which measures the ability of NDV specific antibodies to inhibit the agglutination of red blood cells (RBCs) by the NDV particles (Bello *et.al*, 2018). Besides that, isolation of NDV can also be performed in primary cell cultures which are highly permissive to viruses, then the cells will be infected with clinical samples and monitored for cytopathic effects (CPE) (McGinnes *et.al*., 2006).

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In addition, other diagnostic methods like real-time PCR (qPCR) can be used for detection of viral RNA directly from clinical samples such as swabs, tissues, feces, and discharges from the chicken. RT-PCR can rapidly and accurately detect viral genome in clinical samples with high sensitivity especially if appropriate samples are taken. It is usually designed to simultaneously detect and identify the pathotype of the virus by targeting the Fusion (F) gene portion (Wang *et.al*., 2001). Besides that, serology tests like ELISA are highly sensitive in detecting NDV and produce results that well correlate with HI test results. ELISA platforms utilizing the whole virus as antigens can potentially detect antibodies directed against all the proteins in the NDV particle.

Lastly, to confirm diagnosis, identification of an isolate as vNDV is established by the rapidity of killing day-old SPF chicks inoculated by the intracerebral route, the intracerebral pathogenicity index, or genomic sequencing and identification of a specific amino acid motif at the cleavage site of the fusion protein (F) precursor (F0) (Kiril, 2023).

2.5 PREVENTION AND CONTROL

No medication has been shown to impact the course of virulent NDV infection, and treatment with antivirals is not recommended. In cases of flocks affected by low virulence NDV, supportive care with antimicrobials against secondary infections may help to alleviate clinical signs and decrease morbidity and mortality (Kiril, 2023). However, prevention of NDV is very important to be achieved through biosecurity and vaccination. Firstly, practicing strict biosecurity and implementing strategies to prevent introduction or transmission of NDV into poultry premises are efficient

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preventive and intervention measures. For instance, routine disinfection procedures, feed and water quality, and pest and litter management should be properly controlled to avoid NDV outbreak (OIE, 2021).

Next, vaccines are available for chickens, turkeys, and pigeons and are used to induce an antibody response. There are many types of NDV vaccines such as live attenuated virus vaccine, inactivated virus vaccines and vectored recombinant vaccines. Generally, an excellent NDV vaccine is not only able to prevent clinical disease, but also reduces or abolishes virus shedding and increases the quantity of the virulent virus required to cause infection (Kapczynski *et.al*, 2013). Unfortunately, ND vaccines do not provide sterile immunity, and in many areas of the world, vaccines are used to prevent losses from sickness and death. To be specific, live lentogenic virus vaccines, chiefly B1 and LaSota strains, are the common vaccination regime that is widely used and typically administered to poultry by mass application in drinking water or by spray. Healthy chicks are vaccinated as early as day 1 of life. Mucosal immunity induced in birds vaccinated by live virus vaccines applied by these routes decreases the amount of vNDV but the vaccinated birds may shed if infected with vNDV, compared to the infected chicken vaccinated by inactivated virus vaccine. For inactivated virus vaccines, oil-adjuvanted inactivated virus vaccines are also used after live virus vaccines in breeders and layers. Administering inactivated virus vaccines is safe and low chance of virus mutation but it is more labor intensive due to each bird has to be handled individually besides providing shorter immunity where revaccination or vaccine booster have to be administered to the chickens (Kiril, 2023).

Besides that, vectored recombinant vaccines such as Fowlpox or turkey herpesvirus–vectored NDV vaccines are commercially available for chickens and

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have the advantage of being able to be administered *in ovo* at the hatchery. These vaccines must be reconstituted as directed by the manufacturer. Because they take 3–4 weeks to produce a protective level of immunity, a window of exposure occurs during which chickens are susceptible to vNDV infection and biosecurity becomes even more important (Kiril, 2023).

There are many different types of vaccines that help to prevent the disease but the greatest weakness of the NDV vaccines is their inability to stop the shedding of the heterologous virulent NDV even if clinical protection is achieved. Experimentally, it has been shown that virus shedding can substantially be reduced when birds are immunized with vaccines which are homologous to the challenge strains. So, the new direction in the fight against ND focuses on the generation of the so-called genotype-matched NDV vaccines (Bello *et.al*, 2018). The latest strategy for the development of genotype-matched live attenuated ND vaccines is reverse genetics, which is the recovery of a recombinant virus from its cloned cDNA. There is example of study where Xiao et al. (2012) genetically modified the F cleavage site of a highly virulent NDV circulating in Indonesia and showed that it completely lost its virulence and induces a superb protective immunity that significantly reduced virus shedding following challenge with a highly virulent wild type genotype VII NDV isolate. Besides, another important application of reverse genetic technology is in the generation of marker NDV vaccines able to differentiate vaccinated from the infected animals. Therefore, reverse genetics is an attractive technology for rapid generation of stably attenuated genotype-matched vaccines against virulent NDV (Gururaj, 2014). In short, the most promising vaccines against the virulent NDV infection in poultry are the recombinant genotype-matched live attenuated vaccine candidates generated

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by reverse genetics which specifically target the prevailing genotype in a particular region and are therefore rationally designed to fulfill the criteria of an excellent NDV vaccine (Bello *et al.*, 2018)

2.6 IMMUNE SYSTEM OF EMBRYONATED CHICKEN EGG (ECE)

The embryonated chicken egg (ECE) is a complex structure consisting of an embryo and its supporting membranes which are chorioallantoic membrane (CAM), amniotic and yolk sac. The developing embryo and its membranes provide the diversity of cell types that are needed for the successful replication of a wide variety of different viruses. CAM is the largest of the embryo membranes that enclose the largest cavity within the egg, the allantoic cavity; in the embryonated chicken egg, this cavity contains approximately 5–10 ml of fluid, depending upon the stage of embryonation. Next, the amniotic membrane encloses the embryo and forms the amniotic cavity; in the embryonated chicken egg, this cavity contains approximately 1 ml fluid. The yolk sac is attached to the embryo and contains the nutrients the embryo utilizes during embryonic development and the immediate post-hatch period (Guy, 2015).

Immune system of birds plays a very crucial role in protecting them from infection or disease. Generally, there are 2 main, complementary types of immune defense of chicken which include the nonspecific mechanisms, which act on pathogens in a non-targeted manner via physical and chemical barriers, and components of innate immunity including antimicrobial molecules and cellular mechanisms such as heterophils and macrophages; and the adaptive mechanisms, which target specific pathogens with the production of antibodies from B cells and T lymphocytes (Hincke

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et al., 2019). Due to the development of chicken embryos occurring in an egg chamber that is physically separated from the hen, the egg contains all the required elements to nourish and protect the developing embryo during the entire development cycle prior to hatching. Defensive responses involve the recognition of pathogens by Toll-like receptors (TLRs) present in blood vessels, and by leukocytes that develop within the embryo (heterophils and macrophages).

A complete embryonic development takes 21 days which the chick will hatch after 21 days and usually the first signs of a developing immune system are at ECE D10 (Schilling *et al.*, 2018). There is a study by Kannaki clearly stating that chick embryonic tissues from ECE D3 onwards are able to express TLRs besides recognizing viral ligands and responding to them, thereby exhibiting an innate preparedness. Most TLRs, including TLR2A, TLR3, TLR4, TLR5, TLR7, TLR15 and TLR21 are expressed as early as ECE D3 in the whole chicken embryo preparation (Kannaki *et al.*, 2015). Apart from that, avian heterophils, similar to mammalian neutrophils can provide the first line of defense against bacterial pathogens by releasing microbicidal agents such as reactive oxygen species, proteolytic enzymes and microbicidal peptides from their cytoplasmic granules (Genovese *et al.,* 2013). Heterophils can be found in the blood and gastrointestinal tract of very young chickens compared to older birds (Bar-Shira & Friedman, 2006). Furthermore, innate responses by heterophils can be triggered when PAMPs are engaged with TLRs (Kogut et al., 2005). From a study, heterophils were isolated from 2- and 10-day-old chicks that were in ovo inoculated with CpG-ODN, through the amniotic route at embryonation day ECE D18 or D19, showed increased degranulation when stimulated by killed or live Salmonella enterica serovar Enteritidis (MacKinnon *et al.*, 2009).

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Then, at ECE D4 or D5, there are presence of embryonic macrophages in the blood vessels and at perivascular locations where the chicken embryonic macrophages recognize and phagocytose microbial antigens (Balic *et al.*, 2014). Besides, the yolk sac of the embryonated chicken egg serves as a support for the cells of the innate immune system, including monocytes and macrophages, from ECE D10 and ECE D12, respectively. Yolk sac-derived macrophages in the ECE were shown to enter the developing central nervous system independently of vascularization and their origin was confirmed through the use of quail-chick yolk sac chimeras (Baggott, 2009). And then, on ECE D11 and D12, T cells and B cells are developed, respectively, with B cell differentiation occur ring after ED15. By ED18, the chicken embryo is immunocompetent and capable of producing both an innate and an adaptive response to pathogens (Schilling *et al.*, 2018).

3.0 MATERIAL AND METHODS

3.1 VIRUS

Genotype II NDV LaSota strain with batch number #18301 was retrieved from Malaysian Vaccines and Pharmaceuticals Sdn. Bhd. (MVP). Total of 1.5ml with titre $10^{10.1}$/ml of NDV LaSota strain was obtained and stored in the freezer until the day of virus inoculation into SPF ECE.

3.2 SPECIFIC-PATHOGEN-FREE EMBRYONATED CHICKEN EGGS

A total of 120 2 day-old specific-pathogen-free (SPF) embryonated chicken eggs (ECE) were obtained from Malaysian Vaccines and Pharmaceuticals Sdn. Bhd. (MVP). The eggs were kept in an incubator at 37°C until the age of 3-day old, 5-day

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old and 10-day old ECE to be used to determine the viability of SPF ECE of different ages following inoculation with lentogenic NDV strain LaSota of different inoculation dose. Besides, this study also aims to detect the presence of NDV from the inoculated SPF ECE based on rapid hemagglutination (HA) spot test and quantify the NDV titer from the inoculated SPF ECE based on HA titration test.

Sixty 2-day-old SPF ECE are divided into 12 groups, with each group 5 eggs and inoculated via allantoic cavity according to the following procedure:

Group 1 is Day 3 ECE inoculated with PBS as negative control.

Group 2 is Day 3 ECE inoculated with 10^5 /0.1ml of virus.

Group 3 is Day 3 ECE inoculated with 10^4 /0.1ml of virus.

Group 4 is Day 3 ECE inoculated with 10^3/ml of virus.

Group 5 is Day 5 ECE inoculated with PBS as negative control.

Group 6 is Day 5 ECE inoculated with 10^5 /0.1ml of virus.

Group 7 is Day 5 ECE inoculated with 10^4 /0.1ml of virus.

Group 8 is Day 5 ECE inoculated with 10^3 /0.1ml of virus.

Group 9 is Day 10 ECE inoculated with PBS as negative control.

Group 10 is Day 10 ECE inoculated with 10^5 /0.1ml of virus.

Group 11 is Day 10 ECE inoculated with 10^4 /0.1ml of virus.

Group 12 is Day 10 ECE inoculated with 10^3/0.1ml of virus.

3.3 VIRUS PREPARATION

Before virus preparation, all the tools and materials to be used are sterilized using UV in Biosafety Cabinet Class II. Next, 1.5ml with titre $10^{10.1}$/ml of NDV LaSota strain batch number LaSota #18301 that obtained from MVP were thawed and aliquoted into

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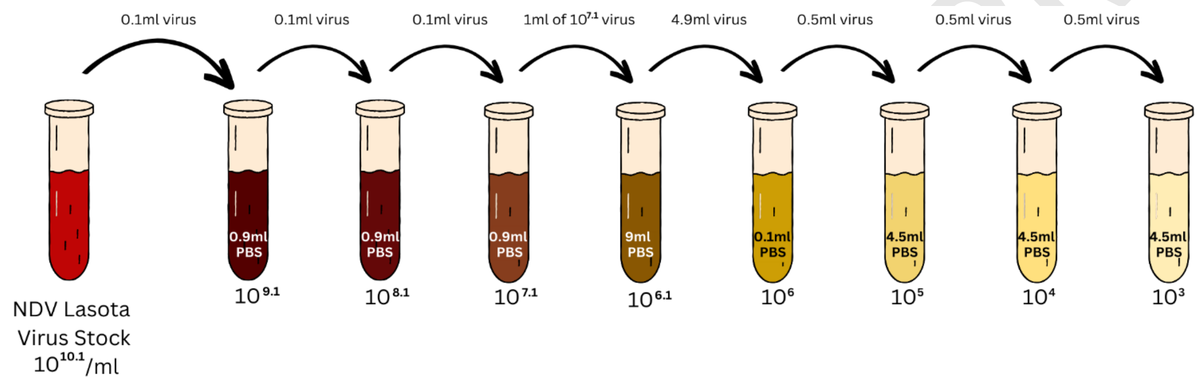
6 small tubes with the amount of 0.2ml NDV LaSota strain/tube. Then, serial dilution of NDV is prepared as shown in Figure 1.

Figure 1: Preparation of serial dilution of NDV LaSota strain



Hence, 3 different inoculation doses of NDV LaSota strain which are 10^5, 10^4 and 10^3 obtained. After that, the dilutions were then filtered using a 0.45 µm syringe filter, for virus inoculation into 3, 5 and 10-days-old SPF ECE.

3.4 VIRUS INOCULATION

Upon NDV inoculation, the surface area of the eggs was disinfected with 70% alcohol. A hole was then punched into each egg using a thumb tack. Next, 0.2mL of PBS was injected into the allantoic cavity of the eggs in the control group. (n=5). 0.2 mL of the viral inoculum (dilution 10-3 to 10-5) was injected into the allantoic cavity of the eggs using a 25G needle (n=5). The holes were then sealed using glue and each egg was labeled accordingly. Then, the inoculated SPF ECE were incubated for 3 days at 37°C with daily candling to monitor the embryonic viability. The dead embryos were kept in the freezer at 4°C, separated from the embryos that remained alive. The embryonic death was recorded daily at 12.30pm, and data was tabulated to determine the viability

of 3, 5 and 10-days old inoculated ECE following inoculation with lentogenic NDV strain LaSota of different inoculation doses.

3.5 HARVESTING ALLANTOIC FLUID OF SPF ECE

To harvest allantoic fluid of the embryonated chicken eggs (ECE), the ECE were chilled overnight at 4°C to kill the embryo and to reduce the contamination of the allantoic fluid with blood during harvesting on day 3 post-inoculation after the embryonic viability of each egg are recorded. Each egg is swabbed with cotton wool with 70% alcohol to disinfect and remove the condensation from the egg shells. Then, a forceps is used to remove the eggshell above the airspace. Next, a micropipette with sterile tip is used to withdraw allantoic fluid from each egg. All the allantoic fluid collected from each egg are placed into sterile containers (Grimes *et.al.,* 2002). Approximately 5-7 ml allantoic fluid of ECE is harvested using for rapid hemagglutination (HA) spot test and HA titration test.

3.6 HAEMAGGLUTINATION(HA) SPOT TEST

HA spot test is used to detect the presence of NDV in the embryonated chicken eggs. Firstly, the allantoic fluid of each dead embryonated chicken egg was harvested and collected into the tube. Next, 50 µL of 1% chicken red blood cells (RBC) were withdrawn using a micropipette (100 µL) and dropped onto a transparent petri dish plate. Then, a micropipette (100 µL) was used to withdraw 50 µL of allantoic fluid from the tubes and then mixed with the 50 µL of 1% chicken red blood cells (RBC) on the plate. After that, the plate was gently rocked to swirl the mixtures of allantoic fluid and RBCs. A positive allantoic sample will give a sandy appearance of RBCs,

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indicating agglutination of the RBCs. Lastly, the results will be recorded (Grimes *et.al.*, 2002).

3.7 HAEMAGGLUTINATION(HA) TITRATION TEST

All strains of Newcastle disease virus will agglutinate chicken red blood cells. This is the result of the haemagglutinin part of the haemagglutinin/neuraminidase viral protein binding to receptors on the membrane of red blood cells. The linking together of the red blood cells by the viral particles results in clumping. This clumping is known as haemagglutination. Haemagglutination is visible macroscopically and is the basis of haemagglutination tests to detect the presence of viral particles. HA titration test is used to quantify the amount of Newcastle disease virus in a suspension. This is done by carrying out two-fold serial dilutions of the viral suspension in a microwell plate and then testing to determine an end point. This result can then be used to determine the amount of haemagglutinin in the suspension and is expressed as a HA titer (Grimes *et.al.*, 2002).

Firstly, 25 µL of PBS is dispensed into each well of the microwell plate. Then, 25 µL of test samples are placed in the first well of each row of column 1. All the samples are tested in triplicate. A multichannel pipette is used to carry out two-fold serial dilutions across the plate until Column 11. Next, 25 µL of 1% red blood cells is added to each well including Column 12. The wells in this column 12 are control wells that contain only PBS and red blood cells. After that, the sides of the plate are gently tapped to mix. The plate is then allowed to stand for 45 minutes at room temperature. The results in each well are read and recorded. For HA negative: a sharp button of red

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blood cells with tear dropped appearance present at the bottom of the V-bottom well. For HA positive: A hazy film of red blood cells, no button or a very small button of red blood cells with no teardrop present at the bottom of the V-bottom well. The endpoint is determined and recorded, and this endpoint will be the last well to show complete haemagglutination and contains one haemagglutinating unit (Grimes *et.al*., 2002).

3.8 STATISTICAL ANALYSIS OF HA TITER OF 3-DAY OLD, 5-DAY OLD AND 10-DAY OLD ECE

In this study, statistical analysis is conducted to compare HA titers of 3-day old, 5-day old and 10-day old ECE following inoculation of NDV LaSota strain using SPSS software. The purpose is to test whether the null hypothesis will be accepted or rejected. The independent variable is the age groups while the dependent variables are HA titer of 3-day old ECE, 5-day old ECE and 10-day old ECE. Total sample size for all inoculated eggs from 3 inoculation doses are 45 eggs, but there are 6 eggs in total that died in first day post-inoculation were excluded due to the early embryonic death is not due to effect of virus but due to non-specific cause of death such as bacteria contamination, injury or damage during inoculation (Orcan, 2020). Hence, the sample size is 39 which is less than 50 sample size. Shapiro-Wilk test was used to test the normality of data before proceeding to parametric or non-parametric test. Parametric tests such as ANOVA will be performed on data sets that fulfil 3 fundamental assumptions which are that data is normally distributed, variances of the population should be approximately equal and lastly there are no extreme scores. If the dataset is

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not normally distributed, non-parametric tests such as Kruskal-Wallis will be used. Lastly, the result will be recorded.

4.0 RESULTS

4.1 EMBRYONIC VIABILITY OF DAY 3, 5 AND 10 SPF ECE FOLLOWING NDV STRAIN LASOTA INOCULATION

The embryonic mortality of all infected ECE with 3 different ages was recorded daily at 12.30pm to determine the viability of SPF ECE of different ages following inoculation with lentogenic NDV strain LaSota of different inoculation dose.

Table 1: Embryonic viability of day 3, 5 and 10 embryonated chicken eggs

	Inoculation dose	Age Group of ECE								
		DAY 3 ECE			DAY 5 ECE			DAY 10 ECE		
		1 dpi	2 dpi	3 dpi	1 dpi	2 dpi	3 dpi	1 dpi	2 dpi	3 dpi
Mortality of Inoculated Eggs	10^3	**0/5**	**4/5**	**5/5**	**2/5**	**5/5**	**5/5**	**0/5**	**1/5**	**1/5**
	10^4	**0/5**	**5/5**	**5/5**	**2/5**	**5/5**	**5/5**	**0/5**	**3/5**	**3/5**
	10^5	**0/5**	**5/5**	**5/5**	**1/5**	**5/5**	**5/5**	**1/5**	**1/5**	**1/5**
	Control	0/5	2/5	3/5	0/5	0/5	0/5	3/5	3/5	3/5
Total no. of death of NDV-inoculated eggs (Control group excluded)		**0/15**	**14/15**	**15/15**	**5/15**	**15/15**	**15/15**	**1/15**	**5/15**	**5/15**

Keywords: days post-inoculation (dpi)

Firstly, for the results of 3-day old SPF ECE (Table 1), all eggs of each dose are alive on day 1 post-inoculation where well-defined blood vessels can be observed

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under a candling lamp. On day 2 post-inoculation, there is only one NDV-inoculated ECE and three ECE of the control group are alive with the presence of blood vessels with slight movement. The remaining ECE (14/15 ECE) that died had disrupted blood vessels and the embryos were small and did not move. Next, on day 3 post-inoculation, all 3-day old infected ECE (15/15 ECE) died and there are 2 eggs from the control group still remained alive with presence of well-defined blood vessels. In overall, the mortality of infected 3-day old ECE increased after 24h post-inoculation.

Next, for the results of 5-day old SPF ECE (Table 1), on day 1 post-inoculation, there are 5 out of 15 ECE died as a result of non-specific death, specifically in inoculation dose 10^3 there are 2 eggs of dose 10^3 died, besides in dose 10^4 there are also two ECE then lastly also in dose 10^5, all ECE are alive except one died with ruptured blood vessels and floating of embryo was observed. On day 2 post-inoculation, all 5-day old ECE of each inoculation dose 10^3, 10^4, 10^5 except control group died and they had disrupted blood vessels and the embryos did not move. Some dead ECE showed a dark ring around the eggs. Next, on day 3 post-inoculation, all control groups of 5-day old ECE were still alive. Hence, all 5-day old ECE of control groups were alive throughout 3 days post-inoculation with sterile PBS. In overall, the mortality of infected 5-day old ECE increased after 24h post-inoculation.

Last but not least, for the results of 10-day old SPF ECE (Table 1), on day 1 post-inoculation, there are one ECE died in dose 10^5 while another 14 ECE remained alive. The ECE that died in 24h post-inoculation is excluded due to non-specific embryonic death. Next, on day 2 post-inoculation, there is total 5 out of 15 ECE died

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which involved one ECE in dose 10^3, 3 out of 5 ECE in dose 10^4 and lastly one ECE in dose 10^5 died showing disrupted blood vessels and the embryos did not move whereas the other remaining ECE including control group were still alive. Lastly, on day 3 post-inoculation, all the ECE that survived on day 2 post-inoculation were remained alive including control group of 10-day old ECE. In overall, the mortality increased slightly after 24h post-inoculation if compared to 3 and 5-day old ECE.

4.2 HA SPOT TEST OF 3-DAY OLD SPF ECE

All the allantoic fluids from the dead embryonated chicken eggs were harvested and HA spot test was done to determine the presence of NDV in the infected 3-day old ECE. After harvest, observation was done on allantoic fluid from each D3 eggs, all ECE appeared cloudy and yellowish. In Table 2, there is one ECE in dose 10^3, 3 out of 5 ECE in dose 10^4 and one ECE in dose 10^5 showed a positive result, where agglutination of the chicken red blood cells (RBCs) can be seen with a sandy appearance in the mixture (Figure 2). The remaining ECE including the control group showed a negative result where there is no agglutination of chicken RBCs (Figure 2). In short, NDV can be detected in day 3 eggs inoculated in all respective doses.

4.3 HA TITRATION TEST OF 3-DAY OLD SPF ECE

For HA positive, a hazy film of red blood cells with a very small button of red blood cells with no tear dropped was observed at the bottom of the V-bottom well (Figure 3). The endpoint which is the last well to show complete haemagglutination and contains one haemagglutinating unit is also determined and recorded. In Table 2, there is one ECE in inoculation dose 10^3, 3 out of 5 ECE in dose 10^4 and one ECE in dose

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10^5 showed a positive result, where the HA titer of that ECE in dose 10^3 is 10^5 and the ECE in dose 10^5 is also 10^5 as well. For HA titer of inoculation dose 10^4, there are two ECE are 10^5, then one ECE is 10^4. The remaining ECE including the control group showed negative results, in which a sharp button of red blood cells with tear dropped appearance were present at the bottom of the V-bottom well (Figure 3). Conclusively, NDV titer of Day 3 eggs in all dose are in a range of 10^4 to 10^5. So, there is no clear evidence of increase in titer corresponding to inoculation dose in Day 3 ECE.

Table 2: Results of HA spot test and HA titration test of day 3 embryonated chicken eggs

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

Keyword: Positive (+), Negative (-)

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Figure 2: HA spot test of 3-days old ECE

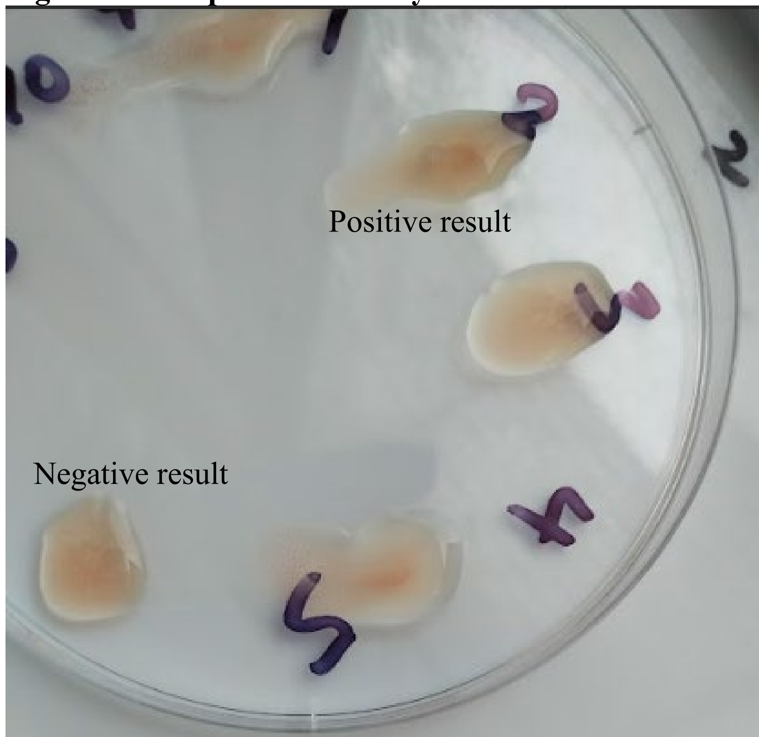
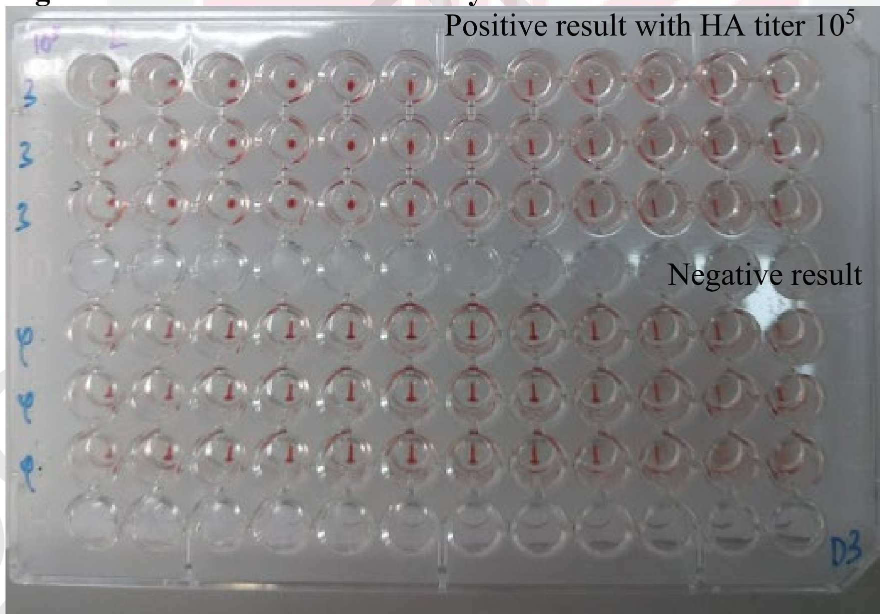


Figure 3: HA titration test of 3-days old ECE



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4.4 HA SPOT TEST OF 5-DAY OLD SPF ECE

After harvest ECE D5, observation done on allantoic fluid collected from each eggs, all appeared cloudy and yellowish except for 3 control ECE D5 eggs, all allantoic fluid collected from 3 control eggs were clear and not yellowish. In Table 3, ECE D5 in dose 10^3 there are 3 out of 5 eggs showed a positive result, all with obvious agglutination that sandy appearance can be seen, then dose 10^4 three eggs also showed positive results with obvious agglutination, lastly dose 10^5 only 2 eggs also showed a positive result, where agglutination of the chicken red blood cells (RBCs) can be seen with a sandy appearance in the mixture (Figure 4). The remaining ECE including the control group showed a negative result where there is no agglutination of chicken EBCs with the absence of a sandy appearance in the mixture (Figure 4). In overall, NDV was also able to be detected by HA spot test on day 5 ECE inoculated in all doses.

4.5 HA TITRATION TEST OF 5-DAY OLD SPF ECE

For HA titration results of 5-day old embryo (Table 3), three eggs of ECE D5 in dose 10^3, three eggs in dose 10^4 and two eggs in dose 10^5 showed a positive result. For the HA titer of dilution 10^3, there are 2 eggs showed titer 10^5, another egg of dose 10^3 is 10^4. Next, for HA titer for dose 10^4, 3 eggs that have positive result all showed different HA titer values which are 10^4, 10^5 and 10^6 (Figure 5). Lastly, HA titer of two eggs in dose 10^5 that showed a positive result is 10^6. The remaining ECE including the control group showed negative results, in which a sharp button of red blood cells with a tear-dropped appearance were present at the bottom of the V-bottom well (Figure 5).

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Hence, there is a trend where HA titers are increasing corresponding to the inoculation doses that are inoculated into eggs.

Table 3: Results of HA spot test and HA titration test of day 5 embryonated chicken eggs

HA spot test						HA titration test					
Inoculation dose	Result (No. of eggs)					Inoculation dose	Result (No. of eggs)				
	1	2	3	4	5		1	2	3	4	5
10^3	+	-	+	-	+	10^3	10^5	-	10^5	-	10^4
10^4	+	-	+	+	-	10^4	10^4	-	10^6	10^5	-
10^5	+	-	-	+	-	10^5	10^6	-	-	10^6	-
Control	-	-	-			Control	-	-	-		

Keyword: Positive (+), Negative (-)

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Figure 4: HA spot test of 5-days old ECE

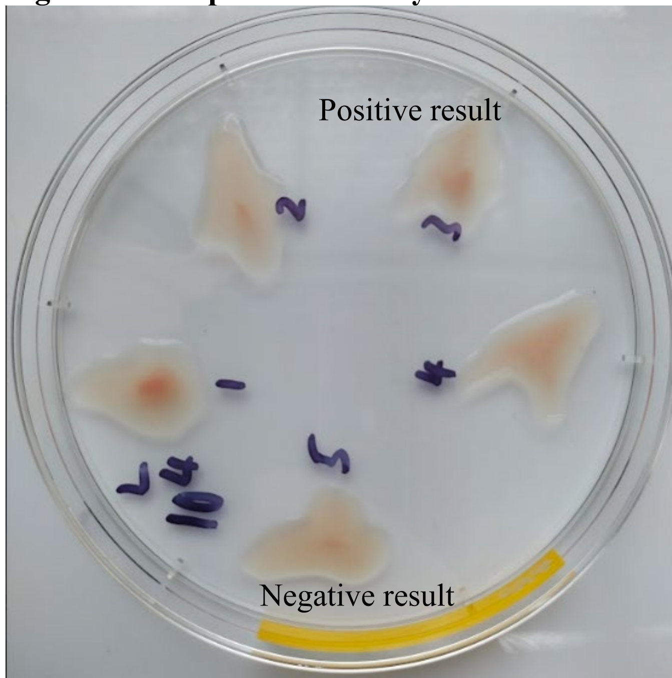
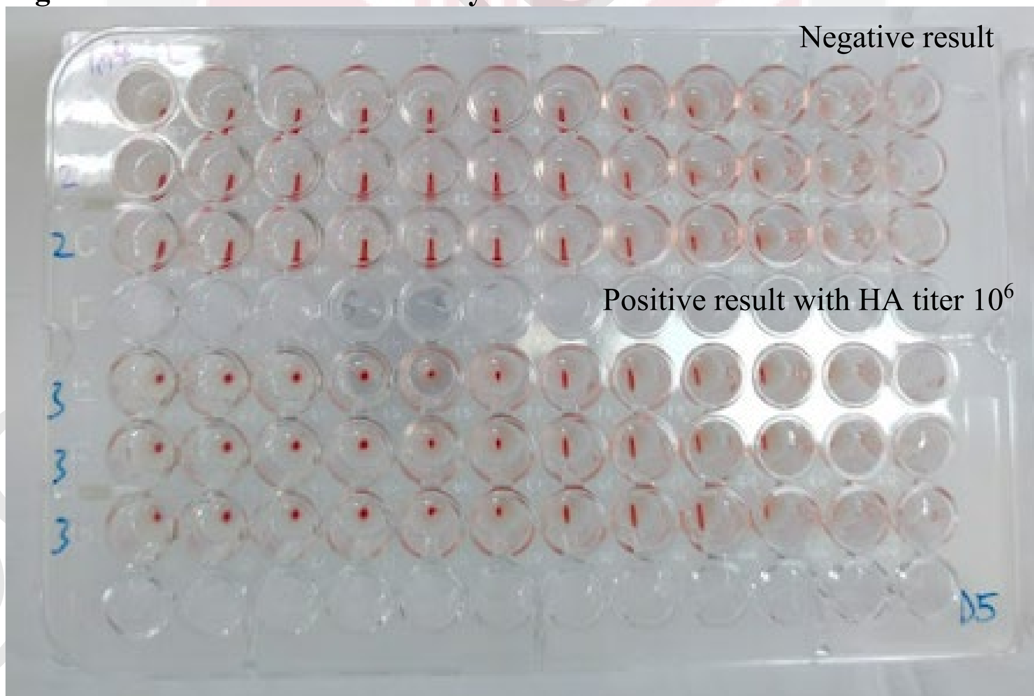


Figure 5: HA titration test of 5-days old ECE



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4.6 HA SPOT TEST OF 10-DAY OLD SPF ECE

After harvest D10 eggs, observation done on allantoic fluid collected from each D10 eggs, all ECE including control group are clear, transparent and not yellowish. In table 4, four ECE D10 in dose 10^3 showed a positive result, all with obvious agglutination that sandy appearance can be seen, then there are two ECE in dose 10^4 showed positive results with obvious agglutination, lastly all eggs in dose 10^5 except one also showed a positive result, where agglutination of the chicken red blood cells (RBCs) can be seen with a sandy appearance in the mixture (Figure 6). The remaining ECE including the control group showed a negative result where there is no agglutination of chicken EBCs with absence of sandy appearance in the mixture (Figure 6). In short, NDV was able to be detected by HA spot test in day 10 eggs inoculated in all dose.

4.7 HA TITRATION TEST OF 10-DAY OLD SPF ECE

For HA titration results of 10-day old embryo (Table 4), four ECE in dose 10^3, two ECE in dose 10^4 and four ECE in dose 10^5 showed a positive result. For the HA titer of ECE in dose 10^3, one ECE is 10^8 and then there are two ECE had HA titer of 10^9, beside the remaining ECE of dilution 10^3 that showed positive result is 10^{10}. Next, for HA titer for dose 10^4, both ECE showed titer 10^9. Lastly, for HA titer of dose 10^5 (Figure 7), one ECE showed 10^{11}, another two ECE are 10^{10} and lastly the last ECE showed 10^9. The remaining ECE including the control group showed negative results, in which a sharp button of red blood cells with tear-dropped appearance were present at the bottom of the V-bottom well. So, there is a trend where HA titers are increasing corresponding to the inoculation dose that inoculated into D10 eggs.

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Table 4: Results of HA spot test and HA titration test of Day 10 Embryonated Chicken Eggs

HA spot test						HA titration test					
Inoculation dose	Result					Inoculation dose	Result				
	No. of Eggs						No. of Eggs				
	1	2	3	4	5		1	2	3	4	5
10^3	+	+	+	-	+	10^3	10^8	10^9	10^9	-	10^{10}
10^4	-	+	-	-	+	10^4	-	10^9	-	-	10^9
10^5	+	+	+	+	-	10^5	10^{11}	10^{10}	10^{10}	10^9	-
Control	-	-	-			Control	-	-	-		

Keyword: positive (+), negative (-)

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Figure 6: HA spot test of 10-days old ECE

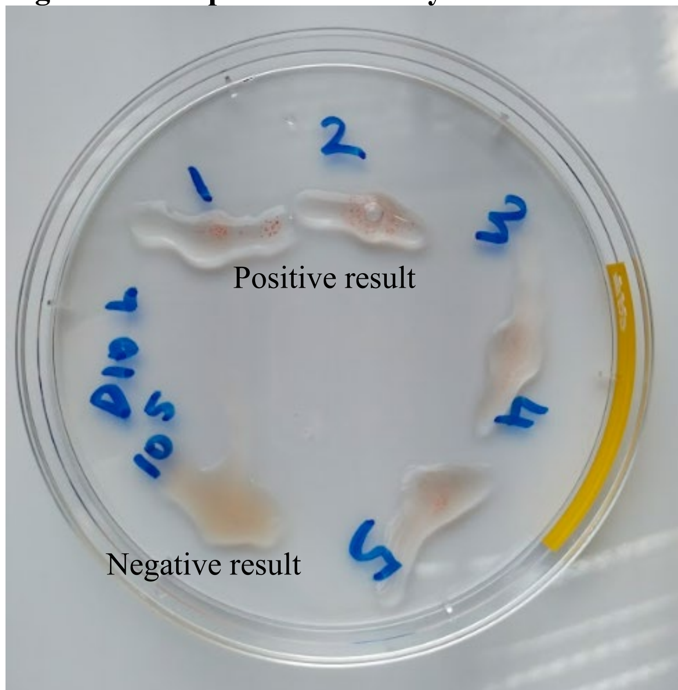
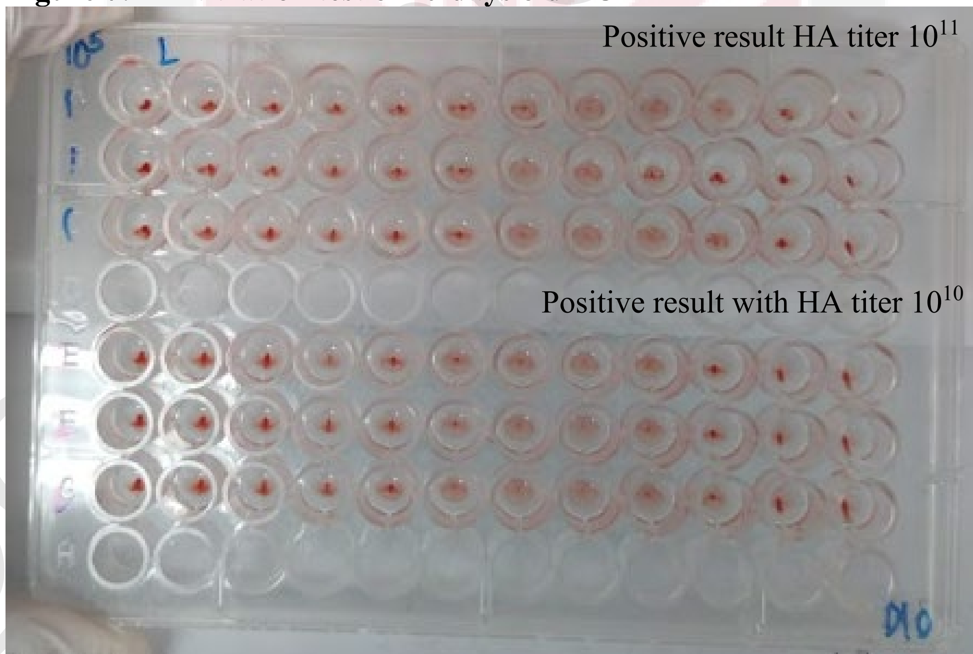


Figure 7: HA titration test of 10-days old ECE



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4.8 STATISTICAL ANALYSIS OF HA TITER OF 3-DAY OLD, 5-DAY OLD AND 10-DAY OLD ECE

Firstly, normality of the data was tested using SPSS software to determine if the data is normally distributed and then decide whether parametric test or non-parametric test to be used. There are various methods available to test the normality of the continuous data, for example, Shapiro–Wilk test, Kolmogorov–Smirnov test, skewness, kurtosis, histogram, and box plot etc. In this study, Shapiro–Wilk test was chosen as the sample size is 39 (Table 5) so it is a more appropriate method for smaller sample sizes (<50 samples). For the result, the null hypothesis states that data are taken from a normal distributed population. When $P > 0.05$, null hypothesis will be accepted and data are called as normally distributed. As a result (Table 6), p-value for HA titer of 3-day old, 5-day old ECE and 10-day old are <0.05. So, the null hypothesis is rejected and the data is not normally distributed.

Table 5: Descriptive Statistic

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
Age_group	45	3	10	6.00	.444	2.977	8.864
HA_Titer	39	0	11	4.08	.622	3.882	15.073
Valid N (listwise)	39						

Table 6: Result of the Shapiro-Wilk test

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Age_group	Statistic	df	Sig.	Statistic	df	Sig.
HA_Titer	3	.418	15	<.001	.623	15	<.001
	5	.283	10	.023	.759	10	.005
	10	.339	14	<.001	.702	14	<.001

a. Lilliefors Significance Correction

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Due to the dataset being not normally distributed, a non-parametric test which is Kruskal-Wallis test is conducted using SPSS software. As a result, p-value is 0.002 which is <0.05, hence indicating the at least of the HA titer of 3-day old, 5-day old and 10-day old are significantly different. Besides that, there are also mean rank presented by the Kruskal-Wallis test where the HA titer of ECE Day 10 has the highest rank, followed by ECE Day 5 then lastly ECE Day 3 has the lowest rank among all ages of embryonated chicken eggs.

Table 7: Kruskal-Wallis test

Test Statistics[a,b]

	HA_Titer
Kruskal-Wallis H	11.999
df	2
Asymp. Sig.	.002

a. Kruskal Wallis Test

b. Grouping Variable: Age_group

Ranks

	Age_group	N	Mean Rank
HA_Titer	3	15	13.00
	5	10	20.60
	10	14	27.07
	Total	39	

5.0 DISCUSSION

Firstly, all age group of ECE were incubated for 3 days with daily monitoring which egg candling was used to determine the embryonic viability. Under the candling lamp, there is presence of blood vessels that are well defined and prominent in a viable embryo. Besides, the embryo that moves in response to the light is also considered as a viable embryo (Figure 8). But, if an embryo has died, the blood vessels start to break down. Then, they appear as streaks under the shell when viewed under the candling lamp. Often deteriorating blood vessels will appear as a dark ring around the egg (Figure 9).

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Figure 8: Example of viable embryos with well-defined blood vessels (black arrow) and moves in response to light (yellow arrow)

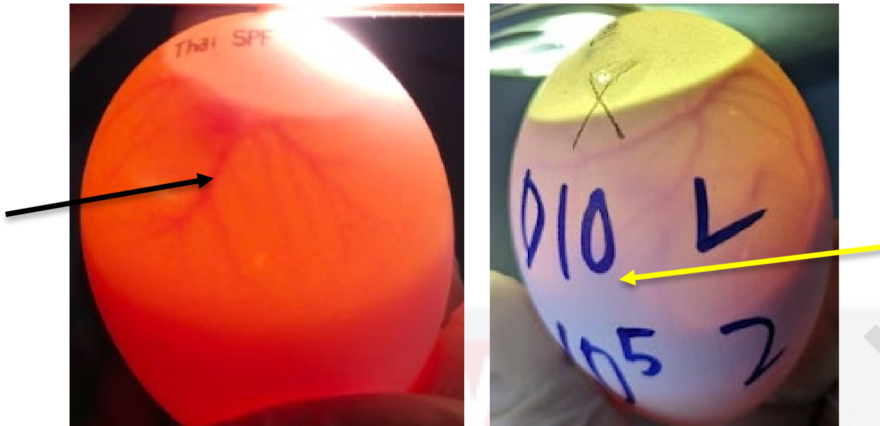

Figure 9: Example of dead embryos with ruptured vessels (black arrowhead) and dark ring appearance around egg (yellow arrowhead)

The embryonic viability of 3, 5 and 10-days old were then recorded. As a result, there are mortality in 5 and 10-days old ECE at 24h post-inoculation. When HA results were analyzed, all 5 and 10-days old ECE that died at 1 dpi had negative HA spot and no HA titer. Hence, it may be caused by non-specific cause of embryonic death, such as bacteria contamination. Guy (2015) stating that embryo deaths that occur <24 h after inoculation generally are due to bacterial contamination, toxicity of the inoculum, or injury. Hence, some diagnostic test to detect bacteria such as Total Plate Count or

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RT-PCR by taking allantoic fluids of ECE as samples can be conducted for further study to better confirm whether embryonic death <24h is due to bacteria contamination.

Next, by comparing the results of embryonic viability in all ages of ECE, mortality of 3-day old and 5-day old ECE increased drastically after 24h compared to 10-day old ECE. This is most probably due to 3-day old and 5-day old ECE are more fragile than 10-day old ECE and they also have incomplete embryonic development compared to 10-day old ECE. Sekellick et al. (1990) stating that cells prepared from young embryos that as early as ED5 still required more time to develop IFN system when cultured in vitro than did cells from older embryos like ED10. Hence, this cause them to be more succumbed to early death that be due to various factors such as stress, bacteria contamination or injury to the embryos during inoculation. Besides that, 10-day old ECE had the highest NDV titer in a range of 10^8 to 10^{11} and also being proved by statistical analysis that day 10 ECE had the highest rank compared to day 3 and 5 ECE, this is because 10-day old ECE is an ideal substrate for viral replication providing with the nutrients required in allantoic fluid (Mansour *et.al.*, 2016). Hence, 10-day old ECE may be the most suitable age to propagate NDV, as 10-day old ECE was able to obtain a very high NDV titer which indicates that high number of viruses are being cultivated to infect host (Qosimah *et.al.*, 2018). Furthermore, 10-day old ECE also able to pursue a lesser early embryonic death compared to 3 and 5-day old ECE which favour NDV replication as virus need living host to survive and replicate.

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6.0 CONCLUSION

Based on statistical analysis, can be concluded that null hypothesis, H0 of this study is rejected, indicating at least one of the NDV titer from day 3, day 5 and day 10 chicken embryos are significantly different. Besides that, all egg of irrespective ages able to support propagation of NDV LaSota strain based on HA spot & titration test. Lastly, day 10 eggs had the lower mortality and the highest NDV titer (10^8 to 10^{11}) compared to Day 3 (10^4 to 10^5) and Day 5 eggs (10^4 to 10^6).

7.0 LIMITATION AND RECOMMENDATION

One of the limitations faced during this study was the challenges to work with early age of embryos as the early age of embryos are more difficult to be handled and also difficult to harvest their allantoic fluids. This might be due to they are more fragile so the yolk sac is more easily to be ruptured and the yolk content was mixed with allantoic fluid, this led to difficulties in harvesting pure and clear allantoic fluid and also increase challenges when interpreting the HA results as the cloudy yolk that mixed with allantoic fluid caused the sandy agglutination in HA spot to be hardly observed. Apart from that, there were many non-specific embryonic mortalities occurred within 24h and it may due to improper inoculation technique, hence must ensure a proper and aseptic inoculation technique to ensure there is no or less embryonic death that due to non-specific cause such as bacteria contamination. Lastly, future study like real-time PCR or immune-related genes study is needed for thorough understanding of the development and functioning of the avian immune system.

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