UNIVERSITI PUTRA MALAYSIA

PATHOTYPING OF A RECENTLY ISOLATED GENOTYPE VII NEWCASTLE DISEASE VIRUS BY MEAN DEATH TIME AND INTRACEREBRAL PATHOGENICITY INDEX

TAN EE LING

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A project paper submitted to the

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Pathotyping of a Recently Isolated Genotype VII Newcastle Disease Virus by Mean Death Time and Intracerebral Pathogenicity Index" by Tan Ee Ling 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, Dr. Nik Mohd Faiz Nik Mohd Azmi, seniors, Fatin Nursyaza Arman Shah, Paniz Zarghami Dastjerdi, Amira Binti Peli, my family, and friends.

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CONTENTS

LIST OF ABBREVIATIONS

NDV	Newcastle disease virus
ND	Newcastle disease
FYP	Final Year Project
ICPI	Intracerebral Pathogenicity Index
IVPI	Intravenous Pathogenicity Index
MDT	Mean Death Time
MLD	Minimum lethal dose
F	Fusion
SPF	Specific-pathogen-free
AOaV-1	Avian *Orthoavulavirus* serotype-1
HN	Hemagglutinin- neuraminidase
ELISA	Enzyme linked immunosorbent assay
HI	Hemagglutination inhibition
FAT	Fluorescent antibody technique
PRNT	Plaque reduction neutralisation test
AGIDT	Agar gel immunodiffusion test
RT-PCR	Reverse transcriptase polymerase chain reaction
IACUC	Institutional Animal Care and Use Committee
MVP	Malaysian Vaccines and Pharmaceuticals
PBS	Phosphate-buffered saline
ECE	Embryonated chicken eggs
RBC	Red blood cell

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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.

PATOTAIP VIRUS PENYAKIT GENOTYPE VII NEWCASTLE MENGIKUT MEAN DEATH TIME DAN INTRACEREBRAL PATHOGENICITY INDEX

Oleh

Tan Ee Ling

2022

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Penyakit Newcastle (ND) ialah penyakit unggas yang dahsyat yang kekal berterusan di seluruh dunia walaupun terdapat pelbagai jenis program vaksinasi. NDV telah dikelaskan kepada 3 patotip: velogenik, mesogenik, dan lentogenik. Selama 2 tahun yang lalu, ladang-ladang komersial di Malaysia telah mengalami wabak genotip VII ND. Pencirian ND virulen yang telah diisolasi baru-baru ini diperlukan untuk membangunkan virus cabaran dalam kajian keberkesanan vaksin. Dalam kajian terdahulu, genotip VII NDV strain UPM008/2021 telah diasingkan dalam telur ayam bebas-patogen-khusus (SPF) dan dicirikan oleh jujukan gen Fusion (F). Satu kajian telah dijalankan

untuk menentukan patotip NDV melalui Masa Kematian Purata (MDT) dan Indeks Kepatogenan Intracerebral (ICPI). MDT telah dijalankan untuk menentukan masa purata untuk dos maut minimum (MLD) untuk menyebabkan 100% kematian embrio. NDV dikelaskan sebagai velogenik, mesogenik, dan lentogenik mengikut MDT pada <60 jam, 60-90 jam, dan > 90 jam, masing-masing. ICPI telah dijalankan untuk menentukan skor purata setiap burung pada setiap pemerhatian selama 8 hari, berdasarkan pemarkahan '0'-sihat, '1'-sakit, dan '2'-mati. Strain velogenik menghasilkan ICPI hampir 2.0, manakala strain lentogenik menghasilkan ICPI hampir atau sama dengan 0. Dalam kajian ini, NDV genotip VII strain UPM008/2021 yang dikaji dikelaskan sebagai NDV velogenik dengan MDT (59.2 jam) dan ICPI (1.75), mengesahkan kajian terdahulu yang menunjukkan virus itu adalah strain velogenik berdasarkan jujukan gen F. Penemuan ini menekankan kepentingan patotip virus dalam membezakan antara strain virus, mengesahkan diagnosis NDV, dan membenarkan penggunaan virus sebagai virus cabaran dalam kajian keberkesanan vaksin.

Kata kunci: *Genotip VII NDV; patotip*; *MDT*; *ICPI*; *Malaysia*

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.

PATHOTYPING OF A RECENTLY ISOLATED GENOTYPE VII NEWCASTLE DISEASE VIRUS BY MEAN DEATH TIME AND INTRACEREBRAL PATHOGENICITY INDEX

By

Tan Ee Ling

2022

Supervisor: Prof. Dr. Abdul Rahman Omar

Co-Supervisor: Dr. Nik Mohd Faiz Nik Mohd Azmi

Newcastle Disease (ND) is a devastating poultry disease that remains persistent worldwide despite intense vaccination programs. NDV can be classified into 3 pathotypes: velogenic, mesogenic, and lentogenic. For the past 2 years, commercial farms in Malaysia have experienced major genotype VII ND outbreaks. Characterisation of recently isolated field virulent ND is necessary for developing challenge viruses in vaccine efficacy studies. In a previous study, genotype VII NDV strain UPM008/2021 was isolated in embryonated specific-pathogen-free (SPF) chicken eggs and characterised by fusion (F) gene sequencing. A study was undertaken to determine the pathotype of the isolated NDV via mean death time (MDT) and

intracerebral pathogenicity index (ICPI). MDT was conducted to determine the average time for the minimum lethal dose (MLD) to cause 100% embryonic mortality. NDV isolate is classified as velogenic, mesogenic, and lentogenic according to MDT at <60 hours, 60-90 hours, and >90 hours, respectively. ICPI was conducted to determine the average score per bird for each observation for 8 days, based on scoring '0'-normal, '1'-sick, and '2'-dead. Velogenic strains produced ICPI near 2.0, while lentogenic strains produced ICPI near or equal to 0. In this study, the genotype VII NDV strain UPM008/2021 is classified as a velogenic NDV with MDT (59.2 hours) and ICPI (1.75), confirming a previous study that indicated the virus is a velogenic strain based on F gene sequencing. These findings emphasise the importance of virus pathotyping in distinguishing between virus strains, confirming NDV diagnosis, and allowing the use of the virus as a challenge virus in vaccine efficacy studies.

Keywords: *Genotype VII NDV; pathotyping*; *MDT*; *ICPI*; *Malaysia*

1.0 INTRODUCTION

Newcastle disease (ND) is a highly contagious viral disease that affects over 250 species of birds of all ages (Alexander, 1997). Newcastle disease virus (NDV), also known as Avian *Orthoavulavirus* serotype-1 (AOaV-1) is a member of the family *Paramyxoviridae* under the genus *Orthoavulavirus* (ICTV, 2019) The disease is classified under list A poultry contagious disease by International des Epizooties (OIE, 2021). The World Organization for Animal Health states that ND is an OIE-notifiable disease when it meets certain criteria of virulence (OIE, 2021).

ND was first reported in 1926 in Java Island, Indonesia (Kraneveld, 1926) and Newcastle-upon-Tyne (Doyle, 1927). Since then, it has become a threat to the commercial poultry industries and backyard poultry farming in terms of clinical and economic consequences, as outbreaks have been continuously reported in many countries (Westbury, 2001). The first ND outbreak in Malaysia was reported in 1934 in poultry flocks in Parit Buntar, Perak (Leow *et al*., 2011).

The transmission of ND primarily occurs through direct contact between infected and healthy birds (Kaleta & Baldauf, 1988). The severity of ND varies, ranging from a peracute disease with almost 100% mortality to a subclinical disease with no lesions. Therefore, it is impossible to identify ND as a single clinicopathologic entity (Cattoli *et al*., 2011). The strains of NDV are classified into a few pathotypes based on the severity of the disease they produce, such as velogenic, mesogenic and lentogenic. The difference in virulence affects the severity of infection. Therefore, determining the virulence of the virus is important for the effective control of ND (Bilal *et al*., 2014).

There are a few pathogenicity tests that can be carried out to determine the pathotypes of NDV isolates, which are divided into in vitro and in vivo studies. In vitro studies include mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI), while in vivo studies include molecular characterisation by F gene sequencing. However, by international agreement, a definitive assessment of virus virulence is based on the intracerebral pathogenicity index (OIE, 2021). The speed of the virus to induce mortality is calculated to determine the virulence of newly isolated NDV.

In the last 2 years, many commercial poultry farms in Malaysia have experienced major Genotype VII ND outbreaks. Genotype VII NDV, primarily subgenotypes VII.2 (VIIh and VIIi) have caused the fifth ND panzootic outbreak that has rapidly spread across (Diel *et al*., 2012; Miller *et al*., 2015). Genotype VII NDV strain UPM008/2021 was isolated in a broiler farm in Selangor. The detection of the isolated virus was confirmed by isolation of the isolated virus in embryonated specific-pathogen-free (SPF) eggs and characterisation by sequencing of fusion (F) gene. The isolated virus needs to be further characterised by assays such as mean death time (MDT) in SPF embryonated eggs and intracerebral pathogenicity index (ICPI) in day-old chicks to determine the virulence of the genotype VII NDV strain UPM008/2021.

1.1 OBJECTIVE

To determine the pathotype of genotype VII Newcastle Disease Virus (NDV) strain UPM008/2021 by pathogenicity tests such as mean death time (MDT) in SPF embryonated eggs and intracerebral pathogenicity index (ICPI) assay in day-old chicks.

1.2 HYPOTHESIS

The null hypothesis (H_0) for this study was:

H_0 = Genotype VII.2 Newcastle Disease Virus (NDV) strain UPM008/2021 has a mean death time of >60 hours with an intracerebral pathogenicity index of < 1.4, classifying it into a mesogenic or lentogenic strain.

The alternative hypothesis (H_A) for this study was:

H_A = Genotype VII.2 Newcastle Disease Virus (NDV) strain UPM008/2021 has a mean death time of <60 hours with an intracerebral pathogenicity index of >1.4, classifying it into a velogenic strain.

2.0 LITERATURE REVIEW

2.1 NEWCASTLE DISEASE VIRUS (NDV)

Newcastle disease virus is a highly contagious viral disease affecting various body systems of domestic poultry and birds. Newcastle disease virus (NDV), also known as Avian *Orthoavulavirus* serotype-1 (AoaV-1) is a member of the family *Paramyxoviridae* under the genus *Orthoavulavirus* (ICTV, 2019). The envelope of NDV has two surface glycoproteins, which are the hemagglutinin-neuraminidase (HN) protein, responsible for virus attachment to the host cell, and fusion (F) protein, which is needed for virus fusion to the host cell membrane (Mahon *et al*., 2011). F and HN proteins are the primary targets of the immune response against NDV, which protects against infection with virulent NDV strains (Seal *et al.*, 2000). NDV has a single serotype, but due to its genetic diversity, it is further divided into many genotypes and subgenotypes using phylogenetic analysis and the nucleotide sequences of the F protein cleavage site (Dimitrov *et al*., 2019). It is a pleomorphic, enveloped virus with a diameter of 200-300 nm. It is a non-segmented, negative sense, single stranded RNA virus with a helical capsid symmetry (Ganar *et al*., 2014). The World Organization for Animal Health states that ND is an OIE notifiable disease when it meets certain criteria of virulence (OIE, 2021).

2.2 NDV CLASSIFICATION

NDV can be classified into a few categories based on the clinical signs observed in infected chickens: velogenic, mesogenic and lentogenic. Strains of NDV in the field vary greatly in the organ systems they impact and the severity of clinical signs they cause in infected birds. The study of virulence and identification of viral determinants of disease severity are crucial to allow a more productive preventative or therapeutic approach for viral infections (Dortmans *et al*., 2011).

Velogenic strains are highly virulent strains that can cause mortality rates up to 100%. It is subdivided into viscerotropic and neurotropic strains. Viscerotropic velogenic strains cause severe hemorrhagic intestinal lesions in the infected chicken, while neurotropic velogenic strains cause high mortality following respiratory and nervous signs (OIE, 2021). Mesogenic strains have intermediate virulence, causing respiratory infection with occasional nervous signs with a 10% mortality rate (Lancester, 1976). Lentogenic strains are low-virulent strains that cause subclinical infection with mild respiratory or enteric diseases.

The identification of NDV isolates from birds exhibiting clinical signs does not confirm a diagnosis of ND due to the extreme variation in virulence of different NDV isolates and the widespread use of live vaccines. Therefore, the isolate's virulence must be evaluated (OIE, 2021). There are different types of assays used to determine the virulence of isolates, which include in vitro studies such as mean death time (MDT) in embryonated SPF eggs, intracerebral pathogenicity index (ICPI) in day-old chicks and intravenous pathogenicity index (IVPI) in six-weeks old chickens, and also in vivo study such as molecular characterisation by F gene sequencing. However, by international agreement, a definitive assessment of virus virulence is

based on the intracerebral pathogenicity index (OIE, 2021). The speed of the virus to induce mortality is then calculated to determine the virulence of newly isolated NDV.

2.3 SOURCE AND TRANSMISSION

NDV can be transmitted to susceptible birds via direct or indirect contact with infected birds. Direct contact, such as contact with respiratory discharges of infected birds through inhalation of aerosols or ingestion via the fecal-oral route. Indirect contact with fomites, such as contaminated food and water, housing, equipments, worker's clothing can also be a source of virus transmission. The presence of feces, as in soiled egg shells, can prolong the survival of NDV. Infected birds can shed viruses during the incubation period. Depending on the host species, birds that survive infection can secrete and excrete the virus in their respiratory secretions and feces for weeks to months. Chickens can shed the virus for 1-2 weeks, while psittacines have been shown to shed NDV intermittently for over one year and have been linked with transmission to the poultry industry (OIE, 2021). Wild birds and waterfowl may serve as reservoir hosts for lentogenic NDV which may become virulent following mutation upon introduction to new host species. Infected chickens, wild birds and carcasses infected with acute NDV infection can act as a source of NDV. The main methods of virus transmission between poultry flocks are the movement of infected birds, and the transfer of the virus, particularly infective feces, by fomites (Aiello & Moses, 2016). The virus is frequently introduced and spread by illegal bird trading and exotic pet bird smuggling. Migratory birds contribute to the virus's natural spatial distribution. Therefore, outbreaks may be seasonal and coincide with migratory behaviours (OIE, 2021).

2.4 CLINICAL SIGNS AND GROSS LESIONS

Clinical signs in birds infected with NDV vary greatly depending on factors such as host age, the strain of NDV, host species, immune status and environmental conditions (Greenacre & Morishita, 2021). Clinical signs alone do not provide a reliable basis for ND diagnosis. The virulence of the virus strain, level of vaccinal immunity, environmental conditions, and flock condition all impact morbidity and mortality (OIE, 2021). The symptoms observed vary depending on whether the infecting virus has tropism on the respiratory, digestive, or nervous systems (Aiello & Moses, 2016). The symptoms can be severe in young birds with little or no maternal antibodies, unvaccinated birds, or under stressful conditions.

NDV can be classified into three pathotypes, velogenic, mesogenic and lentogenic based on the clinical signs observed in infected chickens. Velogenic strains spread quickly through a susceptible flock, causing severe disease and high mortality. Birds are often discovered dead with few or no signs (Greenacre & Morishita, 2021). In unvaccinated birds, morbidity and mortality rates may approach 100% (OIE, 2021). Initial clinical signs seen in infected birds with velogenic form include depression with hyperpnea, lethargy, and inappetence. The birds show progressive loss of strength and prostration. Later on, birds may develop watery greenish diarrhoea. Persistent coughing, gasping for air, and nasal and eye discharge are common signs seen. Cyanosis of the bird's comb and wattle may occur as they turn dark and bluish with the appearance of a swollen head (Greenacre & Morishita, 2021). Birds that survive a severe infection may develop neurologic symptoms such as tremors, torticollis, aberrant circling behavior, tonic-clonic spasms, and a partial or complete cessation of egg production (OIE, 2021).

Clinical signs for the mesogenic form are similar to the velogenic form but less severe with lower mortality rate. Severe symptoms may occur if other co-infectious agents are present. The mesogenic form usually presents with respiratory signs, and nervous signs may occur but are uncommon. A marked drop in egg production can also occur. The lentogenic form usually produces a mild respiratory disease with signs of coughing, sneezing, rales and gasping, together with a sudden drop in egg production. Once the birds recover from the disease, egg production will return back to normal within a few weeks. Mortality is insignificant in lentogenic form. Lentogenic strains are usually used in vaccine production (OIE, 2021).

There are no pathognomonic gross lesions seen in birds infected with NDV. Lesions found on post-mortem vary depending on the infecting virus strain (Greenacre & Morishita, 2021). Several birds must be examined to make a preliminary diagnosis based on the clinical signs, lesions and serological test. Isolation and identification of the causative agent is needed to make a final diagnosis. Velogenic strains produce more significant gross lesions compared to mesogenic and lentogenic strains. The velogenic strain causes congestion and hemorrhages in visceral organs such as the proventriculus, ceca, and small intestine (Morishita, 1996). Hemorrhages may occur in the proventriculus and, less frequently in the small intestines in mesogenic form. The nasal passages, larynx, and trachea are filled with clear fluid. Early cases of the lentogenic form may show no clinical signs or mild tracheitis (Morishita, 1996). Although there are no pathognomonic lesions, ulceration or necrosis of Peyer's patch is suggestive of Newcastle disease (OIE, 2021).

2.5 DIAGNOSIS

A thorough diagnostic investigation is important to differentiate ND from other similar diseases caused by other viral agents, such as Influenza A virus (OIE, 2021). Clinical signs and post-mortem findings of infected birds can aid in diagnosis, however, laboratory diagnosis is required to confirm a disease (Banda, 2002).There are a few laboratory techniques that can be used to diagnose NDV, which are serological testing like hemagglutination inhibition (HI) test, enzyme-linked immunosorbent assay (ELISA), fluorescent antibody technique (FAT), plaque reduction neutralisation test (PRNT) and agar gel immunodiffusion test (AGIDT), virus isolation, molecular techniques such as reverse transcriptase polymerase chain reaction (RT-PCR) (Hasan *et al*., 2012).

Virus isolation is the gold standard for confirming NDV diagnosis as it is the most sensitive method for isolating NDV (Kouwenhoven, 1993; Alexander, 1997). However, it is time-consuming and labour intensive. Viruses can be isolated from recently dead or moribund birds that have been killed humanely. Samples used to isolate NDV are cloacal swabs, feces of dead birds, intestinal contents, tracheal swabs, and bone marrow content (Kouwenhoven, 1993; Alexander, 1997). The virus is then inoculated into the allantoic cavity of 9- to 11-day-old SPF embryonated chicken eggs. The dead embryos that die within 3-6 days after inoculation are tested for hemagglutinating activity. The HA positive samples have to be tested for specific inhibition with antiserum to NDV. A rise in NDV antibody titer measured using hemagglutination inhibition (HI) or ELISA on paired serum samples indicates NDV infection (Aiello & Moses, 2016).

2.6 CONTROL AND PREVENTION

One of the best methods to prevent ND is through vaccination. Birds should be immunised against the local strain of NDV prevalent in each area. Improper vaccination can cause an outbreak of ND (Khan *et al*., 2000; Vyslouzil & Dohnal, 1988; Mustafa & Ali, 2005). There are different routes of vaccinations such as intraocular, intranasal, subcutaneous and drinking water. Vaccines that are used include live NDV strains with low virulence or inactivated strains, and recombinant vectored vaccines (OIE, 2021). Examples of live avirulent strains that are used as primary vaccines are Hitchner-B1, La Sota, Queensland V4, NDW, I2 and F (OIE, 2021). Roakin, Mukteswar and Komarov are mesogenic strains used for secondary vaccinations (OIE, 2021). The antigen dose greatly influences the potency of inactivated vaccines (Maas *et al*., 2000). It has been shown that a combination of live and inactivated vaccine administered simultaneously can provide a better protection against virulent NDV, and has been used in control programs in areas with intensive poultry production (Senne *et al*., 2004). The immune response induced by vaccine is affected by the route of administration (Mutalib & Boyle, 1994). Intraocular and intranasal administration tends to have a longer lasting protection, and maternal antibodies have no effect at the mucosal surfaces of nose, Harderian and paranasal glands. Administrating vaccine through drinking water produces inconsistent results due to the variation of bird water intake. Inactivated vaccines are administrated through intramuscular or subcutaneous route, and are used as secondary vaccinations at the end of rearing period (Kouwenhoven, 1993). To maintain the protective antibody titers, anti-NDV antibody titers are regularly measured and the birds are revaccinated. Nevertheless, good management and hygiene is the foundation for ND prevention. Cleaning and disinfection of farms, restricting access to wild birds and maintaining personal staff hygiene are

essential measures of a hygienic environment (Moerad, 1987). Biosecurity measures such as bird-proof house, restricted access into farm, feed and water supplies, disinfection of vehicles and equipments and pest control should be implemented (Abdisa *et al*., 2017).

3.0 MATERIAL AND METHODS

3.1 ANIMAL ETHICS

The animal trials were conducted under the supervision of the institution's veterinarian and in accordance with the guidelines outlined in UPM's Code of Practice for the Care and Use of Animals for Scientific Purposes. They complied with the current guidelines for animal care and use, which were approved by the Institutional Animal Care and Use Committee (IACUC) under AUP number: UPM/IACUC/AUP-R040/2022 dated 10th August 2022 (Appendix 1). The chicks were euthanised humanely in accordance with the 2013 Edition of the AVMA Guidelines for the Euthanasia of Animals and the 2016 Canadian Code of Practice for the Care and Handling of Farm Animals at the end of the experiment.

3.2 VIRUS

Genotype VII NDV strain UPM008/2021 was isolated in the year 2021 from commercial broilers in a poultry farm in Selangor. The detection of the isolated virus was confirmed by isolation of the virus in embryonated specific-pathogen-free (SPF) eggs and characterisation by sequencing of fusion (F) gene.

3.3 EXPERIMENTAL HOSTS

3.3.1 EXPERIMENTAL DAY-OLD SPF CHICKS

A total number of 20-day-old SPF chicks with mixed gender were purchased from Malaysian Vaccines and Pharmaceuticals Sdn. Bhd. (MVP). The day-old chicks were transferred to Animal Research Facility, Faculty of Veterinary Medicine, UPM and reared in designated rooms for each control and infected group. The handling of chicks was conducted in accordance with laboratory animal care guidelines. The study protocol was approved by the Institutional Animal Care and Use Committee at the Faculty of Veterinary Medicine, UPM (reference no. UPM/IACUC/AUP-R040/2022) dated 10th August 2022.

3.3.2 EMBRYONATED SPF CHICKEN EGGS

A total of 110 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 10 day old to be used for pathotyping of NDV by calculation of the mean death time (MDT) of the egg embryos.

3.4 PATHOGENICITY TESTS

3.4.1 INTRACEREBRAL PATHOGENICITY INDEX

The intracerebral pathogenicity index (ICPI) is the average score of daily observation of chick health over an 8-day period. The pathotype of the UPM008/2021 isolate was determined using ICPI. In brief, fresh infective allantoic fluid with a HA titer $>2^4$ (>1/16) was diluted 1/10 into sterile phosphate-buffered saline (PBS) with no additives added such as antibiotics. A sample size of 20 day-old SPF chicks aged over 24-hours and under 40-hours old were used. 0.05ml of the diluted virus was injected intracerebrally into 10 chicks, while another group of 10 chicks were injected with 0.05 ml PBS intracerebrally to act as control group. The chicks were examined for clinical symptoms every 24 hours for 8 days, and they were scored at each observation where '0'- normal, '1'- sick, and '2'- dead. Birds that were still alive but unable to eat or drink were humanely culled and scored as dead at the next observation. Total scores were recorded, and the mean daily score was calculated to obtain the ICPI, which is the mean score per bird per observation over the 8-day period (OIE,2021) NDV isolates were classified as velogenic strains with an ICPI of 1.5-2.0, mesogenic with an ICPI of 0.5-1.5, and lentogenic with an ICPI <0.5 (OIE, 2021).

3.4.2 MEAN DEATH TIME

Mean death time (MDT) is the average time in hours for the minimum lethal dose (MLD) to kill all the inoculated SPF embryonated chicken eggs (ECE). The minimum lethal dose is the highest virus dilution that can cause 100% embryonic mortality. For the control group and each virus dilution, a sample size of 10 day-day-old SPF ECE were used. Five eggs for each virus dilution and control group were used for the morning session, while the remaining 5 eggs were used for the afternoon session. All the SPF ECE were candled to see the embryonic viability and a 'X' was marked at the base of the air sac. The surface area of the eggs were disinfected with 70% alcohol. A hole was punched into each egg using a thumb tack. A ten-fold serial dilution of fresh infective allantoic fluid UPM008/2021 was prepared in 1x sterile PBS. (300 µL of allantoic fluid into 2700 µL PBS to give a ten-fold dilution series) (10^{-1} to 10^{-10}). The dilutions were then filtered using a 0.45 µm syringe filter. 0.1 mL of the viral inoculum (10^{-1} to 10^{-10}) was injected into the allantoic cavity of the eggs using a 25G needle (n=5). 0.1mL of PBS was injected into the allantoic cavity of the eggs in the control group. (n=5) The holes were then sealed using glue. The infected SPF eggs were then incubated at 37°C for the morning session. The same procedures were repeated 8 hours later in the afternoon. The inoculated SPF ECE were incubated for 5 days at 37°C with daily candling to monitor the embryonic death. The embryonic death time was recorded twice daily at 9 am & 5 pm, and data was tabulated to calculate the MDT based on the OIE standard manual. NDV isolates were classified as velogenic, mesogenic, and lentogenic according to MDT at <60 hours, 60-90 hours, and >90 hours, respectively.

3.5 HA SPOT TEST

HA spot test is a direct and macroscopic test that detects agglutination of chicken red blood cells (RBCs). The allantoic fluid of the dead embryonated chicken eggs was harvested. A micropipette was then used to collect 50 μL of allantoic fluid from the eggs and dropped onto a white ceramic plate. 50 μL of 5% chicken red blood cells (RBC) were added to each of the allantoic fluid samples and mixed. 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, indicating agglutination of the RBCs.

4.0 RESULTS

Intracerebral Pathogenicity Index

The intracerebral pathogenicity index (ICPI) is the average score of daily observation of chick health over an 8-day period. On day 1 post-inoculation of the diluted isolated virus in the infected group, 5 out of 10 SPF chicks showed clinical signs, whereas the remaining 5 were still healthy. The chicks that showed clinical signs appeared dull and depressed (Figure 1). On day 2 post-inoculation, 5 of the chicks were found dead while 5 were showing clinical signs such as watery greenish diarrhea (Figure 2), nasal discharge (Figure 3), and swelling of the head (Figure 4). On day 3 post-inoculation, all the remaining chicks in the infected group died (Figure 5), with a mortality rate of 100% within 3 days. For the control group, all 10 SPF chicks remained healthy during the 8-day period. The chicks were scored at each observation for clinical symptoms every 24 hours for 8 days with the scoring system based on '0'- normal, '1'- sick, and '2'- dead. The scores were recorded and tabulated (Table 1).

CLINICAL SIGNS	**DAY 1**	**DAY 2**	**DAY 3**	**DAY 4**	**DAY 5**	**DAY 6**	**DAY 7**	**DAY 8**	**Total**	**Score**
NORMAL	5	0	0	0	0	0	0	0	5 x **0**	0
SICK	5	5	0	0	0	0	0	0	10 x **1**	10
DIE	0	5	10	10	10	10	10	10	65 x **2**	130
									Total	**140**

Table 1: Recording of scores of chicks over an 8-day period.

The mean daily score was then calculated based on OIE to obtain the ICPI. NDV isolates were classified as velogenic strains with an ICPI of 1.5-2.0, mesogenic with an ICPI of 0.5-1.5, and lentogenic with an ICPI <0.5 (OIE, 2021).

Based on the calculation:

$$Intracerebral\ Pathogenicity\ Index\ (ICPI) = \frac{(5x0) + (10x1) + (65x2)}{80}$$

$$= 1.75$$

Hence, the pathogenicity study classified the Genotype VII NDV strain UPM008/2021 as a velogenic strain with an ICPI value of 1.75.

Figure 1: Day 1 post-inoculation of virus. Infected chick appeared dull and depressed.

Figure 2: Day 2 post-inoculation of virus. Watery greenish diarrhea was seen on floor housing.

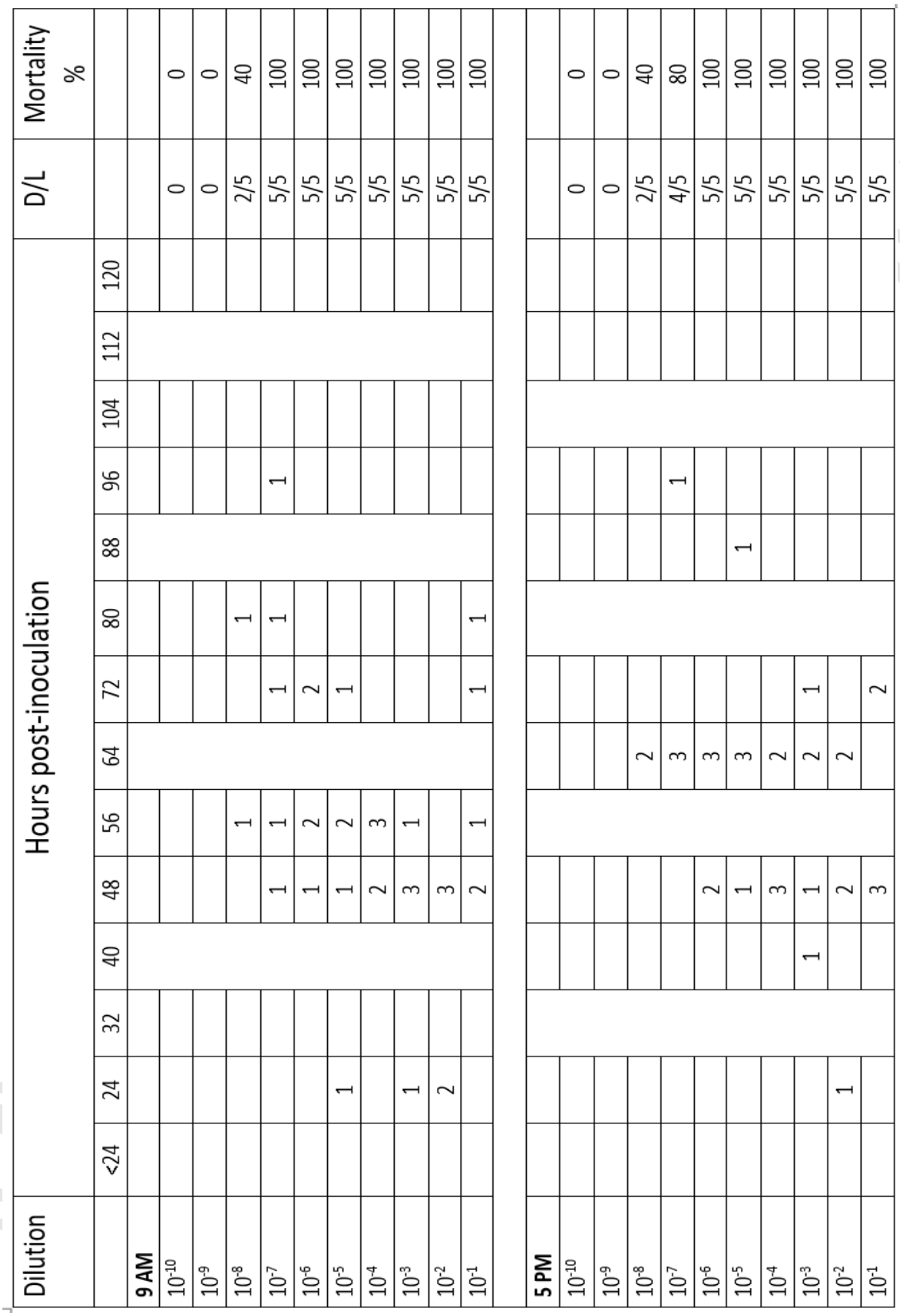
Figure 3: Day 2 post-inoculation of virus. Nasal discharge seen in infected chicks.

Figure 4: Day 2 post-inoculation of virus. Swelling of head seen in infected chicks.

Figure 5: Day 3 post-inoculation of virus. The remaining chicks in the infected group have died.

Mean Death Time

Mean death time (MDT) is the average time in hours for the minimum lethal dose (MLD) to kill all the inoculated SPF embryonated chicken eggs (ECE). NDV isolates can be classified as velogenic, mesogenic, and lentogenic according to MDT at <60 hours, 60-90 hours, and >90 hours, respectively. The inoculated SPF ECE were incubated for 5 days at 37°C with daily candling to monitor the embryonic death. The embryonic death time was recorded twice daily at 9 am & 5pm, and data was tabulated to calculate the MDT based on the OIE standard manual (Table 2).



Dilution	Hours post-inoculation												D/L	Mortality %
	<24	24	32	40	48	56	64	72	80	88	96	104	112	120		
9 AM																
10^{-10}															0	0
10^{-9}															0	0
10^{-8}						1			1						2/5	40
10^{-7}					1	1		1	1		1				5/5	100
10^{-6}					1	2		2							5/5	100
10^{-5}		1			1	2		1							5/5	100
10^{-4}					2	3									5/5	100
10^{-3}		1			3	1									5/5	100
10^{-2}		2			3										5/5	100
10^{-1}					2	1		1	1						5/5	100
5 PM																
10^{-10}															0	0
10^{-9}															0	0
10^{-8}							2								2/5	40
10^{-7}							3				1				4/5	80
10^{-6}					2		3								5/5	100
10^{-5}					1		3			1					5/5	100
10^{-4}					3		2								5/5	100
10^{-3}				1	1		2	1							5/5	100
10^{-2}		1			2		2								5/5	100
10^{-1}					3			2							5/5	100

Table 2: Recording of embryonic death time at 9 am & 5 pm daily for 5 days.

Mean death time is calculated based on the formula:

$$Mean\ Death\ Time = \frac{time\ (hours) for\ MLD\ to\ cause\ 100\%\ embryonic\ mortality}{total\ no. of\ chicks\ in\ MLD}$$

NDV isolates are classified as velogenic, mesogenic, and lentogenic according to MDT at <60 hours, 60-90 hours, and >90 hours, respectively. Based on the results tabulated, the minimum lethal dose of the isolated virus is 10^{-6}, which caused 100% embryonic mortality.

Based on the calculation:

$$Mean\ Death\ Time\ (MDT) = \frac{(3x48) + (2x56) + (3X64) + (2x72)}{10}$$

$$= 59.2\ hours$$

Therefore, the pathogenicity study classified the Genotype VII NDV strain UPM008/2021 as a velogenic strain with an MDT of 59.2 hours.

HA SPOT TEST

All the allantoic samples from the dead embryonated chicken eggs 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).

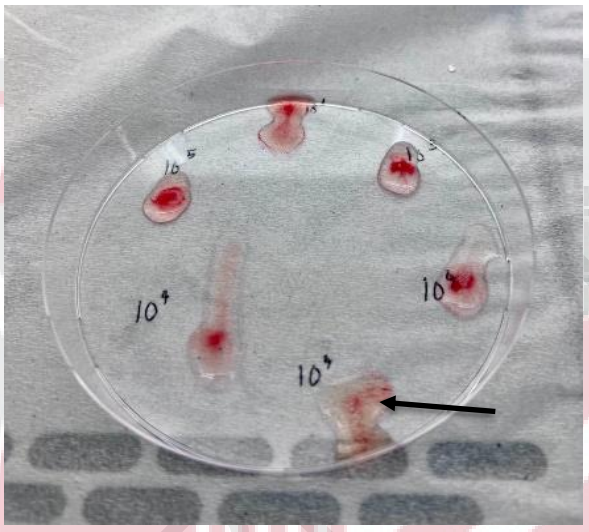


Figure 6: Positive HA spot test. (black arrow)

5.0 DISCUSSION

ND is a devastating poultry disease that remains endemic in Malaysia since its first detection in 1934 despite intense vaccination programs and mass culling. Genotype VII ND has been the predominant strain, causing ND outbreaks in commercial poultry farms around Malaysia since the 2000s. ND is an economically important poultry disease as poultry meat contributes to the primary protein source for human consumption. (Shohaimi *et al*., 2015).

In the present study, Genotype VII NDV strain UPM008/2021, isolated in a broiler farm in Selangor, was detected and confirmed through virus isolation in embryonated specific-pathogen-free (SPF) eggs and characterisation by sequencing of fusion (F) gene. The amino acid residues at the F gene cleavage site had identified the isolated virus with a polybasic motif, indicating a velogenic virulence. Pathogenicity tests such as Mean Death Time (MDT) and Intracerebral Pathogenicity Index (ICPI) was conducted to further characterise Genotype VII NDV strain UPM008/2021. Based on ICPI, the current study found that Genotype VII NDV strain UPM008/2021 had velogenic strain characteristics with an ICPI value of 1.75. Based on MDT, the current study found that the isolated virus had velogenic strain characteristics with a MDT value of 59.2 hours. Therefore, Genotype VII NDV strain UPM008/2021 which was isolated from a broiler farm in Selangor during the year 2021 is a velogenic strain virus. HA spot test was conducted to detect agglutination of chicken red blood cells (RBCs) commonly used for selected viruses that have hemagglutinin protein such as NDV, however, it does not identify the presence of the etiological agent present in the samples tested. Positive HA spot test results when hemagglutinin on NDV surface binds to the chicken RBCs, causing agglutination which gives a sandy appearance of RBCs.

Virus ID	Location	Types of Birds	MDT	ICPI
UPM/NDV/IBS303/2016	Penang	Broiler	58.2	1.7
UPM/NDV/IBS362/2016	Sabah	Broiler	58.4	1.7
UPM/NDV/IBS380/2017	Perak	Broiler	56.2	1.7
UPM/NDV/IBS501/2017	Kedah	Broiler	57.6	1.7
UPM/NDV/IBS559/2017	Penang	Broiler	57.0	1.7
Genotype VII NDV strain UPM008/2021	Selangor	Broiler	59.2	1.75

Table 3: NDV isolated from different locations around Malaysia and different time periods.

Previous pathogenicity studies based on MDT and ICPT on NDV isolated from ND cases in different states in Malaysia had similar pathogenicity as the Mean Death Time (MDT) values ranged less than 60 hours, whereas the Intracerebral Pathogenicity Index (ICPI) value ranged more than 1.5 (Mahamud *et al*., 2021) (Table 3). The results of the present study agreed with that obtained by Mahamud *et al*., (2021) where the viruses isolated were of a velogenic virulence. This suggests that the current trend of ND infection circulating in Malaysia are caused by velogenic strain viruses.

6.0 CONCLUSION

Based on the calculation of the Mean Death Time and Intracerebral Pathogenicity, the pathotype of Genotype VII Newcastle Disease Virus (NDV) strain UPM008/2021 was determined. The isolated virus had a Mean Death Time of 59.2 hours with an Intracerebral Pathogenicity Index of 1.75, classifying it as a velogenic strain. Pathotyping of the virus allows the differentiation between virus strains, confirms the diagnosis of NDV, and enables the isolated virus to be used as a challenge virus in the vaccine efficacy study.

7.0 LIMITATIONS AND RECOMMENDATION

One of the constraints of this study was that further characterisation of the isolated virus using techniques such as complete genome sequencing was not able to be conducted due to a time constraint. Complete genome sequencing would take a much longer time to complete. Another possible suggestion of this study is to perform other assays to further characterise the isolated virus is to perform intravenous pathogenicity index (IVPI) in six-week-old chickens.

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