UNIVERSITI PUTRA MALAYSIA

EFFICACY STUDY OF GENOTYPE MATCHED NEWCASTLE DISEASE VIRUS VACCINE BETWEEN EYEDROP AND DRINKING WATER VACCINATION IN SPECIFIC-PATHOGEN-FREE CHICKENS

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Efficacy Study of Genotype Matched Newcastle Disease Virus Vaccine between Eyedrop and Drinking Water Vaccination in Specific-Pathogen-Free Chickens" by Tan Iee Hong and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement of the course VPD 4999-Project.

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DW	: Drinking water
EID	: Embryonic infectious dose
ED	: Eyedrop
ELISA	: Enzyme-linked immunosorbent assay
HI	: Hemagglutination Inhibition
MVP	: Malaysian Vaccines and Pharmaceuticals
NDV	: Newcastle diseases virus
NVNDV	: Neurotropic velogenic Newcastle diseases virus
PBS	: Phosphate buffered saline
pc	: post- challenged
PCR	: Polymerase chain reaction
SPF	: Specific-Pathogen-Free
VVNDV	: Viscerotropic velogenic Newcastle diseases virus

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901 -Projek.

KAJIAN KEBERKESANAN VAKSIN PADANAN-GENOTIP PENYAKIT NEWCASTLE ANTARA TITISAN MATA DAN VAKSINASI AIR MINUMAN DALAM AYAM BEBAS-PATOGEN-SPESIFIK

Oleh

Tan Iee Hong

2022

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Penyakit Newcastle (ND) adalah penyakit yang sangat berjangkit yang menyebabkan kematian sehingga 100% pada burung. Vaksin yang dipadankan dengan genotip didapati berkesan dalam mengawal ND dalam ayam komersial. Kajian ini bertujuan untuk menentukan keberkesanan vaksin NDV dipadankan genotip (mIBS025) berikutan suntikan titisan mata (ED) dan air minuman (DW) dalam ayam bebas-patogen-khusus (SPF). Kajian ini dijalankan ke atas 135 ekor anak ayam SPF berumur sehari yang dibahagikan kepada 9 kumpulan. Kumpulan 1 adalah kumpulan yang tidak divaksinasi, kumpulan 2 hingga 5 telah divaksinasi sekali melalui ED pada usia 14 hari, manakala kumpulan 6 hingga 9 telah

divaksin melalui DW dua kali pada umur 1 dan 14 hari. Dos untuk kedua-dua laluan yang divaksin berkisar antara $10^{4.5}$ dose berjangkit embrio (EID) $_{50}$, $10^{5}EID_{50}$, $10^{5.5}EID_{50}$, dan $10^{6}EID_{50}$. Pada usia 35 hari, semua ayam tersebut telah dicabar melalui laluan intranasal dengan $10^{6}EID_{50}$ dengan virus ND velogenik genotip VII.2 strain UPM008/2021. Tiada simptom klinikal diperhatikan dalam semua kumpulan selepas sehari dicabar . Dalam kumpulan yang tidak divaksinasi, tanda-tanda klinikal ringan diperhatikan pada hari ke-2, dan pada hari ke-3 dan ke-4 selepas dicabar, ayam menunjukkan tanda-tanda klinikal sederhana seperti mata bengkak, kerutan dan kemurungan dan bertambah teruk pada hari ke-5 dan seterusnya. Kadar kematian kumpulan yang tidak divaksin bermula pada hari ke-5 dan pada hari ke-9 selepas dicabar, 13 daripada 15 ayam telah mati (87%). Kadar kematian turut mencapai 100% pada hari ke-12 selepas dicabar. Dalam kumpulan yang divaksinasi, tiada kematian direkodkan dan hanya seekor ayam dalam kumpulan ED menunjukkan tanda-tanda klinikal ringan mata bengkak. Pemeriksaan bedah siasat ke atas ayam mati menunjukkan lesi kongesi umum dan pendarahan pada trakea dan paru-paru. Kesimpulannya, vaksinasi ED tunggal dan dua dos vaksin DW dengan vaksin ND yang dipadankan dengan genotip, mIBS025 boleh memberikan perlindungan lengkap terhadap cabaran dengan genotip VII ND dalam ayam SPF.

Kata kunci: air minuman, ayam bebas-patogen-khusus, dose berjangkit embrio, titisan mata, virus penyakit Newcastle.

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4901- Project.

EFFICACY STUDY OF GENOTYPE MATCHED NEWCASTLE DISEASES VIRUS VACCINE BETWEEN EYEDROP AND DRINKING WATER VACCINATION IN SPECIFIC-PATHOGEN-FREE CHICKENS

by

Tan Iee Hong

2022

Supervisor: Prof. Dr. Abdul Rahman Omar

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

Newcastle disease (ND) is a highly contagious disease that causes up to 100% mortality in birds. A genotype-matched vaccine was found to be effective in controlling ND in commercial chickens. This study aims to determine the efficacy of genotype-matched NDV vaccine (mIBS025) following eye drop (ED) and drinking water (DW) vaccination in specific-pathogen-free (SPF) chickens. The study was conducted on 135 SPF day-old chicks that were divided into 9 groups. Group 1 was the non-vaccinated group, groups 2 to 5 were vaccinated once via ED at 14 days old,

while groups 6 to 9 were vaccinated via DW twice at 1 and 14 days old. The doses for both vaccinated routes range from $10^{4.5}$ embryonic infectious dose $(EID)_{50}$, $10^{5}EID_{50}$, $10^{5.5}EID_{50}$, and $10^{6}EID_{50}$.

At 35 days old, all the birds were challenged via an intranasal route with $10^{6}EID_{50}$ with velogenic genotype VII.2 NDV strain UPM008/2021. No clinical symptoms were observed in all the groups 1-day post challenge (pc). In the unvaccinated group, mild clinical signs were observed on day 2 pc, and by day 3 and 4 pc, the birds were showing moderate clinical signs such as swollen eyes, ruffles and depression that became severe on day 5 pc onwards. The mortality rate of the unvaccinated group started on day 5 pc and by day 9 pc, 13 out of 15 birds had died (87 %). The mortality rate reached 100% at day 12 pc. In the vaccinated group, no mortality was recorded, and only one chicken in the ED group showed mild clinical signs of swollen eyes. Post-mortem examination of the dead chickens showed generalised congestion, and tracheal and lung haemorrhage. In conclusion, single ED vaccination and two doses of DW vaccination with genotype-matched ND vaccine, mIBS025 can confer complete protection against challenges with genotype VII ND in SPF chickens.

Keywords: drinking water, embryonic infectious dose, eyedrop, Newcastle diseases virus, specific-pathogen-free chickens

1.0 Introduction

Newcastle disease virus (NDV), also known as Avian Orthoavulavirus serotype-1 (AOAV-1) is an important virus that causes an economically important poultry disease known as Newcastle disease (ND) affecting poultry industry. The virus is a single stranded, non-segmented, negative-sense RNA virus (ICTV, 2019). Regarding animal population, poultry is the world's largest domestic animal stock (Otte *et al.,* 2012). Before the emergence of the highly pathogenic Asian H5N1 influenza virus, the impact of virulent NDV was unmatched by any other poultry virus and was likely to have a bigger impact on the global economy than any other animal virus (Alexander *et al.,* 2013). The disease's devastation is greater in developing nations where traditional poultry farming is predominant and serves as a significant source of income and animal protein for households (Dzogbema *et al.,* 2021).

There are over 240 species of birds that can be infected by the NDV which is primarily spread by direct contact between sick and healthy birds (Kaleta and Baldauf, 1988). It is mostly infecting poultry flocks among avian species, and chickens are the most vulnerable, while ducks and geese are the least (Vyslouzil and Dhonal, 1988). The disease is characterised by affecting the respiratory, nervous, gastrointestinal, and reproductive systems (Suarez *et al.,* 2019). The first report of NDV came from Indonesia in 1926 and New Castle upon Tyne, England, in 1927 (Doyle, 1927; Kraneveld, 1926). The first ND outbreak in Malaysia was reported in 1934 in Parit Buntar, Perak (Leow *et al.,* 2011). NDV has been reported in all the continents around the world. As of today, the fifth panzootic is currently spreading quickly across Asia

and the Middle East, and strains with genotype VII NDV are among the most commonly isolated. Thus, genotype VII NDV is a significant threat that must be addressed to ensure the global poultry industry's maximum productivity (Diel *et al.,* 2012; Miller *et al.,* 2015). Based on the virulence and severity of NDV, the strains can be classified into three pathotypes: lentogenic, mesogenic, and velogenic. Velogenic strains are further subdivided into viscerotropic and neurotropic velogenic strains (Dortmans *et al.,* 2011). According to the variation in pathotypes of NDV, the rate of mortality and morbidity in a flock is variable (Haque *et al.,* 2010). The presence of NDV can be confirmed by virus isolation and serological assays such as the Hemagglutination Inhibition (HI) Test, enzyme-linked immunosorbent assay (ELISA), and molecular diagnostic tests such as real-time polymerase chain reaction (PCR) (Munir *et al.,* 2014).

The current NDV vaccines have been in use for more than 50 years and have a solid proven record of efficacy and safety. Even though many countries maintain a stringent vaccination policy against ND, there are indications that ND outbreaks can still occur. There are a few reasons that might contribute to poor immunity development after vaccination. One possible explanation may be due to antigenic divergence between the vaccine and circulating field strains (Dortmans *et al.,* 2012). All the commercially available vaccine strains belong to genotypes I and II (Mahamud *et al.,* 2022) while most of the outbreak in Asia is caused by genotype VII NDV (Miller *et al.,* 2015). These strains are of genotypes I and II, and they are 18.3% to 26.6% genetically distant from common strains of genotype VII (Dimitrov *et al.,* 2016). Thus, having genotype-matched vaccines that are more effective than genotype I and II vaccines,

especially in reducing the load and duration of virus shedding post-challenge is one of the ways to stop the spread of ND (Yang *et al.,* 2017; Sedeik *et al.,* 2019; Sultan *et al.,* 2020). Genotype-matched vaccines have been shown to demonstrate higher protective efficacy, but the route for the vaccine to obtain higher efficacy is not known. Hence, the objective of the study is to compare the protective efficacy of genotype-matched NDV vaccine mIBS025 after single eyedrop vaccination and double drinking water vaccination.

The hypotheses of the study are:

- H0 = Double drinking water vaccination with the genotype-matched NDV vaccine mIBS025 has less efficacy than single eyedrop vaccination.
- HA = Double drinking water vaccination with the genotype-matched NDV vaccine mIBS025 has higher efficacy than single eyedrop vaccination.

2.0 Literature Review

2.1 Newcastle disease virus

Newcastle disease virus (NDV), also known as Avian Orthoavulavirus serotype-1 (AOAV-1) belongs to the genus Orthoavulavirus, subfamily of Paramyxovirinae, and family of Paramyxoviridae (ICTV, 2019). The virus is classified using a system that makes use of the entire F gene's coding sequences and incorporates several objective criteria for identifying NDV, such as phylogenetic topology, inter-populational evolutionary nucleotide distances, branch support, and the epidemiological independence of at least four isolates per subgenotype (Diel *et al.,* 2012). The use of this system and criteria resulted in the classification of class I NDV isolates into a single genotype (genotype 1) with three sub-genotypes, whereas class II viruses were classified into 15 genotypes (I to XV) with multiple sub-genotypes (Diel *et al.,* 2012a). This system was widely used and resulted in the identification of three additional genotypes (XVI, XVII, and XVIII) (Courtney *et al.,* 2013; Snoeck *et al.,* 2013b).

The first report of NDV came from Indonesia in 1926 and New Castle upon Tyne, England, in 1927 (Doyle, 1927; Kraneveld, 1926). NDV outbreaks are constantly reported from around the world. As of today, the fifth panzootic is currently spreading quickly across Asia and the Middle East, which is primarily caused by genotype VII and subgenotype VII.2 (VIIh and VIIi) (Diel *et al.,* 2012; Miller *et al.,* 2015).

2.2 Clinical signs

Newcastle disease (ND) is one of the most serious infectious diseases that affect poultry. It is widely distributed and has the potential to cause significant economic losses in the poultry industry (Lancaster, 1976). The incubation period of the NDV ranges from 2 to 15 days, with an average of 5 to 6 days (Hanson and Spalatin, 1978). Clinical signs often appear in chickens on the second day after disease onset. The birds will then stop eating and become dull on the third day, chicks will become severely depressed and inactive with hard ruffled feathers on the fourth day, and open mouth breathing will begin on the fifth day after the disease onset (Kommer *et al.,* 2002).

Depending on the virus strain, NDV can cause a variety of symptoms affecting the digestive system, nervous system, respiratory system, and reproductive system of affected birds, resulting in up to 100% morbidity and mortality (Suarez *et al.,* 2019). The common respiratory signs that can be observed include sneezing, gasping for air, nasal discharge and coughing (Kommer *et al.,* 2002). The infected chickens also experienced nervous symptoms, including paralysis of the wings and legs, head and neck twisting, torticollis, or complete paralysis (Bhaiyat *et al.,* 1994). Digestive signs include decreased feed and water intake and copious greenish white diarrhoea (Kommer *et al.,* 2002). Clinical signs of the reproductive system include a decrease in egg production, misshapen eggs, rough or stumpy shelled eggs, and a decrease in albumen quality (Ashraf *et al.,* 2018). In addition to the clinical signs mentioned above, other common clinical signs on chickens also include depression, ruffled feathers, oedema

around the eyes, and swelling of the head (Dzogbema *et al.,* 2021). Compared to mature birds, young birds often show more severe and acute signs (Alexander, 2003).

Based on the virulence and severity of NDV, the strains can be classified into three pathotypes, such as lentogenic, mesogenic, and velogenic. Velogenic strains are further subdivided into viscerotropic and neurotropic velogenic strains (Dortmans *et al.,* 2011).

A Viscerotropic Velogenic Newcastle Diseases Virus (VVNDV) strain tends to cause acute, lethal infections with 100% mortality (Moura *et al.,* 2016). The strain affects the digestive system of the chicken, resulting in copious greenish white diarrhoea that quickly leads to dehydration and collapse of the chickens (Dzogbema *et al.,* 2021). The clinical sign normally starts with listlessness, increased respiration, and weakness, ending with prostration and death (Saif *et al.,* 2008). Infected birds normally die within one or two days. Birds that survived the initial phase frequently present with nervous symptoms (Abdisa and Tagesu, 2017).

Neurotropic Velogenic Newcastle Diseases Virus (NVNDV) strains normally induce respiratory and neurological signs, but without gut lesions (Alexander, 2000). It has 100% morbidity but only 50% mortality and limited virus spread when compared to VVNDV (Saif *et al.,* 2008). Sudden depression and inappetence can be seen along with coughing and other respiratory tract symptoms, followed by nervous signs that develop within a few days after the disease onset (Abdisa and Tagesu, 2017). The nervous signs normally become obvious between 5 and 14 days post-infection (Brown

*et al.,*1999). A drop in egg production can also be observed but the diarrhoea sign is usually absent (Saif *et al.,* 2008).

Mesogenic strains cause respiratory distress with occasional nervous signs and tend to have lower mortality due to their intermediate virulence (Dzogbema *et al.,* 2021). However, young and susceptible birds are more vulnerable to mortality (Saif *et al.,* 2008). Other clinical signs that are frequently observed also include depression, weight loss, and decreased egg production for up to three weeks (Abdisa and Tagesu, 2017). The disease caused by mesogenic strains only results in 25% mortality (Marin *et al.,*1996).

Lentogenic strains are considered to have low virulence and usually do not cause disease in adult birds (Saif *et al.,* 2008). The disease is typically associated with infection in younger birds with subclinical to mild respiratory infections (Moura *et al.,* 2016). There may be a slight decrease in egg production and nervous signs are usually absent, with negligible mortality (Abdisa and Tagesu, 2017).

2.3 Transmission

Oral and respiratory transmissions are two of the routes that NDV diseases can spread from diseased to healthy poultry (Sharif *et al.,* 2014). The infected chicken shed a large amount of virus in their faeces, nasal discharge, lacrimal discharge, and exhaled air (Nawanta *et al.,*2008). Even clinically healthy, vaccinated birds that show no clinical signs can still shed the virus after exposure (Abdisa and Tagesu, 2017). This virus will

be transmitted to the healthy chickens through inhalation of secretions from infected animals' respiratory tracts and mouths, ingestion of infected animal's faeces or airborne transmission (Dzogbema *et al.,* 2021). The virus can be carried in the air and spread around within the confined area. It has been demonstrated that the virus is capable of travelling 64 metres and infecting the area (Hanson and Spalatin, 1978). Environmental conditions such as humidity also plays an important role in the outbreak of NDV through airborne transmission (Hugh *et al.,*1973)

Besides, there is also indirect transmission, where the virus can spread through contaminated water, feed, transport and husbandry equipment. Farm workers serve as an intermediary to spread the virus through their clothing and footwear if they are not sanitised or disinfected properly (Dzogbema *et al.,*2021).

2.4 Gross lesion

The strain and pathotype of the infecting virus determine the gross lesions and the organs affected in birds with NDV (Saif *et al.,* 2008). The presence of haemorrhagic lesions in the intestine of infected chickens allows for the differentiation between the viscerotropic velogenic strain and the neurotropic velogenic strain (Hanson, 1980). Necropsies of birds infected with the VVNDV reveal haemorrhagic lesions in the digestive tract (Dzogbema *et al.,* 2021). These lesions are frequently prominent in the mucosa of the proventriculus, ceca, small and large intestine (Abdisa and Tagesu, 2017). Additionally, the cervix and Peyer's patches also exhibit prominent haemorrhaging,

which is primarily caused by lymphoid or intestinal wall necrosis (Saif *et al.,* 2008). Gross lesions are typically not seen in the central nervous systems of birds infected with VVNDV (McFerran and McCracken, 1988). Egg yolk peritonitis are common in chickens infected with velogenic viruses. The ovarian follicles are frequently degenerating and flaccid. There may also be haemorrhage and discoloration of the other reproductive organs (Saif *et al.,* 2008).

For the neurotropic velogenic strains, haemorrhage is normally observed along with the secretion of purulent exudates from the bronchioles (Dzogbema *et al.,* 2021). The respiratory tract signs are predominantly mucosal haemorrhage and marked congestion of the trachea (Alexander and Allan, 1974). Air sacculitis as well as thickening of the air sacs with catarrhal or caseous exudates, are frequently seen in conjunction with secondary bacterial infections in the neurotropic velogenic strain (Beard and Hanson, 1984). Due to the ability to invade and replicate in neural tissue, this pathotype is frequently associated with neuronal degeneration. This includes central neurological lesions that involve the cerebellum, brain stem, midbrain, and spinal cord (Dzogbema *et al.,* 2021).

2.5 Vaccine administration routes

Vaccination is one of the most effective methods of preventing ND outbreaks. Farmers use a few common routes to administer vaccines to chickens, which include eye drop (ED), intra-nasal spray, subcutaneous injection, and drinking water (DW)

(Sharif *et al.,* 2014). By comparing the most common routes of vaccination, the intraocular route produce a better immunological response than the DW vaccination. This is due to the presence of the Harderian gland, which is located behind the eyes. The gland plays an important role in the production of local and humoral immunity. Meanwhile, in oral vaccination, some vaccine viruses can be destroyed by gastric secretions, leading to lower immunity development. (Okwor *et al.,* 2013). Besides, there is also less uniform uptake and more frequent application requirements of the DW vaccination. The vaccine must be given twice, the first time 2-3 weeks apart, and then every three months thereafter (Getabalew *et al.,*2019). The advantages of DW vaccination, are that it requires less labour and produces less stress for the birds. In intraocular route the vaccination titre given to each chicken is more accurate and uniform (Thekisoe *et al.,* 2004). The route allows the vaccine to be delivered into the Harderian gland just behind the eye, which in chicken is a crucial organ for the formation of the immune response, therefore, offers good protection for the chicken (Getabalew *et al.,*2019). However, it requires a lot of labour to catch the chicken (Thekisoe *et al.,*2004).

2.6 Newcastle diseases control

Prevention is always the best control for Newcastle Diseases (ND), as there is no cure for the disease. Primary vaccination best to use to control the virus as it reduces mortality significantly (Dzogbema *et al.,* 2021). Although available vaccines provide

protection against morbidity and mortality, several studies have shown that they do not prevent infection and virus shedding, which could lead to the transmission of the virus to poorly vaccinated birds (Miller *et al.,* 2009). The virus-shedding period in vaccinated birds can still range from 4 months (Utterback and Schwartz, 1973) to 12 months (Allan et al., 1980).

Besides, biosecurity also plays an important role in preventing the outbreak of ND. During the construction of new poultry farms, proper distances should be maintained between poultry farms. A broiler poultry farm should be built 1 km away from a nearby poultry farm, and a breeder farm should be built 3 km away. The strict biosecurity at the poultry farm aids in the prevention of viral and bacterial diseases (Sharif *et al.,* 2014). The movement of people within and out of the farm should be strictly monitored. Personnel equipment must also be sanitised and kept clean at all times (Saif *et al.,* 2008). Other important biosecurity measures also include disinfecting vehicles that enter the farm and control of pests (Abdisa and Tagesu, 2017).

Lastly, good hygiene management is also important in preventing outbreaks of disease on poultry farms. Excess humidity and faecal droppings on feeding trays, drinkers, inside egg-laying nests and windows, accumulation of bad air in poultry houses and other factors contribute to an unsanitary environment. This may increase the contamination of the surrounding area with viruses or bacteria. Thus, to prevent ND, litter should be completely dry, and good hygiene measures should be implemented (Sharif *et al.,* 2014).

3.0 Materials and Methods

3.1 Chickens

This study was approved by the International Animal Care and Use Committee (IACUC): AUP-U030/2022. A total of 135 specific-pathogen-free (SPF) day-old chicks were obtained from Malaysian Vaccines and Pharmaceuticals (MVP) to use in the study. The study was conducted at the Animal Research Facility, Faculty of Veterinary Medicine, Universiti Putra Malaysia. The 135 SPF day-old chicks were divided into 9 groups. Group 1 was the non-vaccinated group. Group 2 to 5 were vaccinated once via ED vaccination and Group 6 to 9 were vaccinated via DW twice. The doses for both vaccinated routes range from $10^{4.5}$ EID_{50}, $10^{5}EID_{50}$, $10^{5.5}EID_{50}$, and $10^{6}EID_{50}$.

3.2 Vaccination Program

The NDV vaccine used in the experiment was mIBS025 which was provided by MVP. For both vaccination routes, the vaccines were prepared in 4 different dosages ranging from $10^{4.5}EID_{50}$, $10^{5}EID_{50}$, $10^{5.5}EID_{50}$, and $10^{6}EID_{50}$.. Group 1, also known as the control group, received sterile saline through the DW route as part of their vaccination programme at a day old.

Group 2 to 5 were vaccinated once via ED vaccination at 14 days old. The ED vaccination was prepared by diluting 10^{9} EID_{50}/mL of vaccine virus with phosphate buffered saline (PBS) with a dilution factor of 1:100 and 1:1000 in order to produce vaccine viruses of 10^{7} EID_{50}/mL and 10^{6} EID_{50}/mL respectively. To achieve a vaccination dose of 10^{6} EID_{50}/0.1mL and 10^{5} EID_{50}/0.1mL for the birds respectively,

0.1 mL was taken from both of the vaccine viruses and administered to the birds. Then, the 10^9 EID_{50}/mL of vaccine virus was diluted again with PBS with a dilution factor of 1:3.16 to achieve a vaccination dose of $10^{8.5}$ EID_{50}/mL of vaccine virus. A dilution factor of 1:100 and 1:1000 was then used to further dilute the vaccine virus to obtain a vaccination dose of $10^{6.5}$ EID_{50}/mL and $10^{5.5}$ EID_{50}/mL of vaccine virus. 0.1 mL was then taken from both vaccine viruses and administered to the birds to achieve a vaccination dose of $10^{5.5}$ EID_{50}/0.1mL and $10^{4.5}$ EID_{50}/0.1mL respectively. The vaccination doses for Group 2, 3, 4 and 5 are 10^6 EID_{50} , $10^{5.5}$ EID_{50}, 10^5EID_{50} and $10^{4.5}$ EID_{50}, respectively.

Group 6 to 9 were vaccinated twice via DW vaccination at 1 day and 14 days old. The vaccine was prepared by mixing 1mL of 10^9 EID_{50} vaccine virus in the form of allantoic fluid with 499mL of ultrapure water. The drinker containing 290 mL of ultrapure water was then filled with 10 mL of the mixture to create the final quantity of 300 mL of drinking water, which contained 10^6 EID_{50} of the vaccine virus. For obtaining vaccine titer of $10^{5.5}$ EID_{50}, 10^5EID_{50} and $10^{4.5}$ EID_{50}, vaccine virus of 10^9 EID_{50} /mL, $10^{8.5}EID_{50}$/mL and 10^8EID_{50}/mL were diluted in PBS with a dilution factor of 1:3, respectively, to obtain $10^{8.5}EID_{50}$/mL, 10^8EID_{50}/mL and $10^{7.5}EID_{50}$/mL vaccine virus. These vaccine viruses were then titrated with similar steps and prepared as above to obtain vaccination doses of $10^{5.5}$ EID_{50}, 10^5EID_{50} and $10^{4.5}$ $EID_{50,}$ respectively. The vaccination doses for Group 6, 7, 8 and 9 are 10^6 EID_{50}, $10^{5.5}$ EID_{50}, 10^5EID_{50} and $10^{4.5}$ $EID_{50,}$ respectively.

3.3 Virus challenge study

All 135 SPF chickens were given an intranasal challenge with a velogenic genotype VII.2 NDV strain UPM008/2021, at the age of 35 days. The virus was obtained from the Institute of Bioscience at UPM. PBS was then used to titrate the virus by using a 1:100 dilution from 10^8 EID_{50} to 10^6 $EID_{50.}$ The challenged virus was transported in a well-packed ice cooler container in order to preserve the virus. Using a pipette, 0.1mL of the challenge virus was administered to each bird.

3.4 Assessment of vaccination efficacy

Clinical signs were closely monitored twice a day for two weeks after challenge for evidence of respiratory signs, nervous signs and gastrointestinal signs. In order to compare whether the single ED vaccination method or the double DW vaccination method can provide better protection, the morbidity and mortality rates of the chickens were assessed for two weeks after the virus challenge. Post-mortem were conducted on the dead chickens to evaluate and observed the development of gross lesions in the organs.

4.0 Results

4.1 Post-challenged clinical signs

After the chickens from all the groups were challenged with the virus, only the control group exhibited a variety of clinical signs. On the first day post challenge (dpc), the morbidity rate for all the groups remained 0% as none of the challenged chickens were showing clinical signs (Table 4.1). Later, chickens from Group 1 (control group) showed clinical signs at 2 dpc where 13% (2/15) of the chickens had swollen eyes. At 3dpc, the chickens from the control group that exhibited clinical signs increased from 13% to 67% (10/15) with clinical signs of swollen eyes and depression. The diseases worsen in 4dpc, with a morbidity rate of 100% (15/15) and symptoms such as nasal discharge, ruffled feathers, swollen eyes, greenish white diarrhoea and depression were observed (Figure 4.1). Among the chickens in the control group, all of them showed clinical signs such as swollen eyes, depression, and diarrhoea. Only four of them showed nasal discharges, and six of them showed ruffled feathers.

On the other hand, the mortality rate of the control group chickens was recorded at 27% (4/15) at 5dpc and the rate increased to 67% (10/15) at 6 dpc. The rate continued to rise steadily, reaching 87% (13/15) at 7dpc, 93% (14/15) at 9dpc, and 100% (15/15) at 12dpc (Table 4.2). Meanwhile, the chickens that went through the single ED and double DW vaccination programmes did not exhibit any clinical signs. Both the vaccinated groups also showed no mortality.

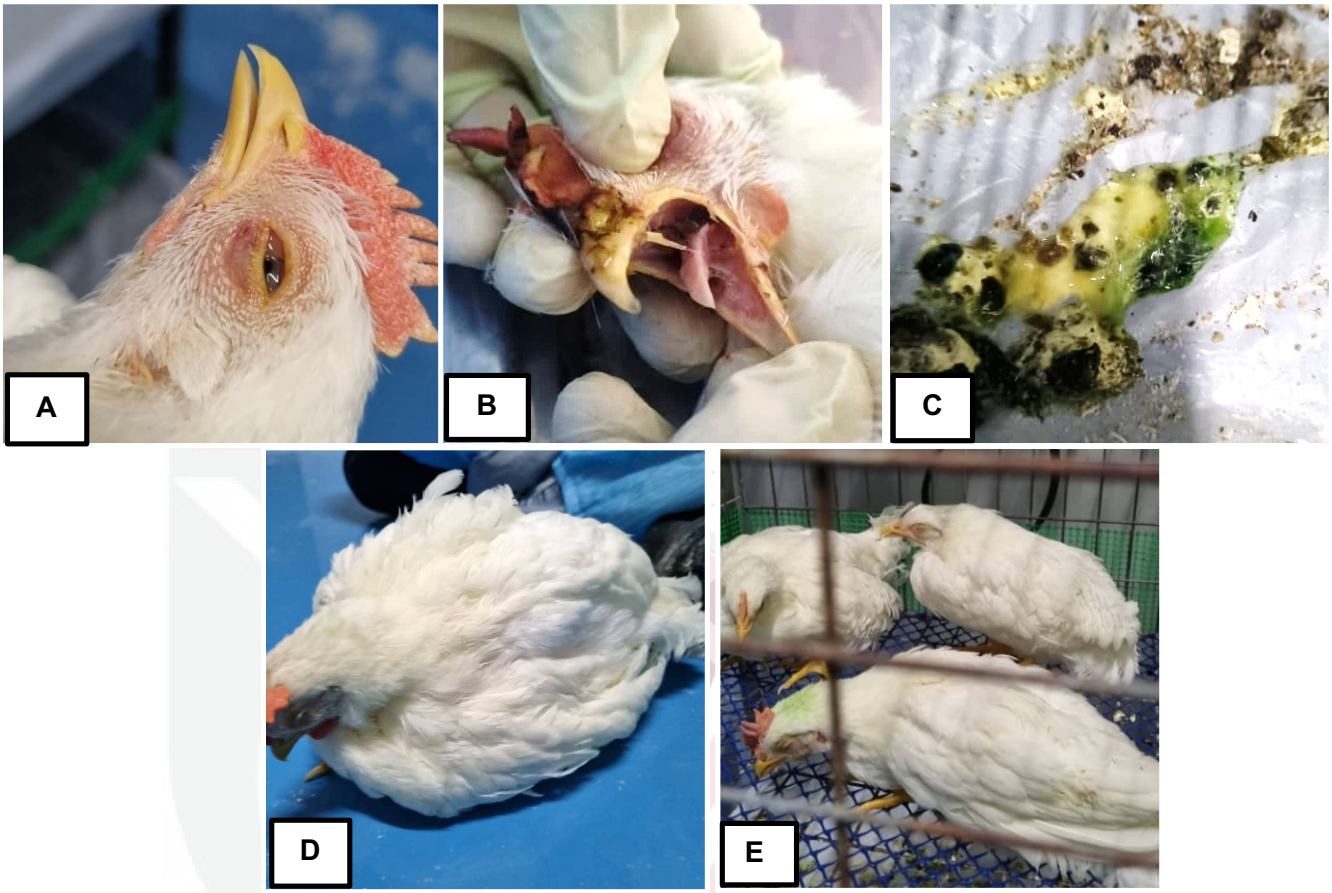

Figure 4.1 : *Challenged chickens from Group 1(control group) showed clinical signs such as A) swollen eyes, B) nasal discharge, C) greenish white diarrhoea, D) ruffled feathers, E) depression.*

Table 4.1 : *Daily morbidity rate of chickens post-challenged in all groups.*

Day post challenged	**Morbidity of the chickens (Chicken exhibits with clinical signs / Total number of chickens)**		
	Control Group (G1)	**ED groups (G2 – G5)**	**DW groups (G6 – G9)**
1	0/15	0/15	0/15
2	2/15	0/15	0/15

3	10/15	0/15	0/15
4	15/15	0/15	0/15

Table 4.2 : *Daily mortality rate of chickens post-challenged in all groups.*

Day post challenged	**Mortality of the chickens (Chicken mortality number / Total number of chickens)**		
	Control Group (G1)	**ED groups (G2 – G5)**	**DW groups (G6 – G9)**
1	0/15	0/15	0/15
2	0/15	0/15	0/15
3	0/15	0/15	0/15
4	0/15	0/15	0/15
5	4/15	0/15	0/15
6	10/15	0/15	0/15
7	13/15	0/15	0/15
8	13/15	0/15	0/15
9	14/15	0/15	0/15
10	14/15	0/15	0/15
11	14/15	0/15	0/15
12	15/15	0/15	0/15

4.2 Post-challenged gross lesions

A post-mortem examination of the dead chickens from the control groups was performed to look for the development of gross lesions. Only four of the fifteen

chickens that died developed post-mortem lesions such as mucus secretion in the respiratory tract and trachea and haemorrhage on the lungs and trachea (Figure 4.2) The rest of the chickens had no significant lesions observed upon post-mortem except for general congestion. Only the respiratory system was involved in the development of gross lesions.

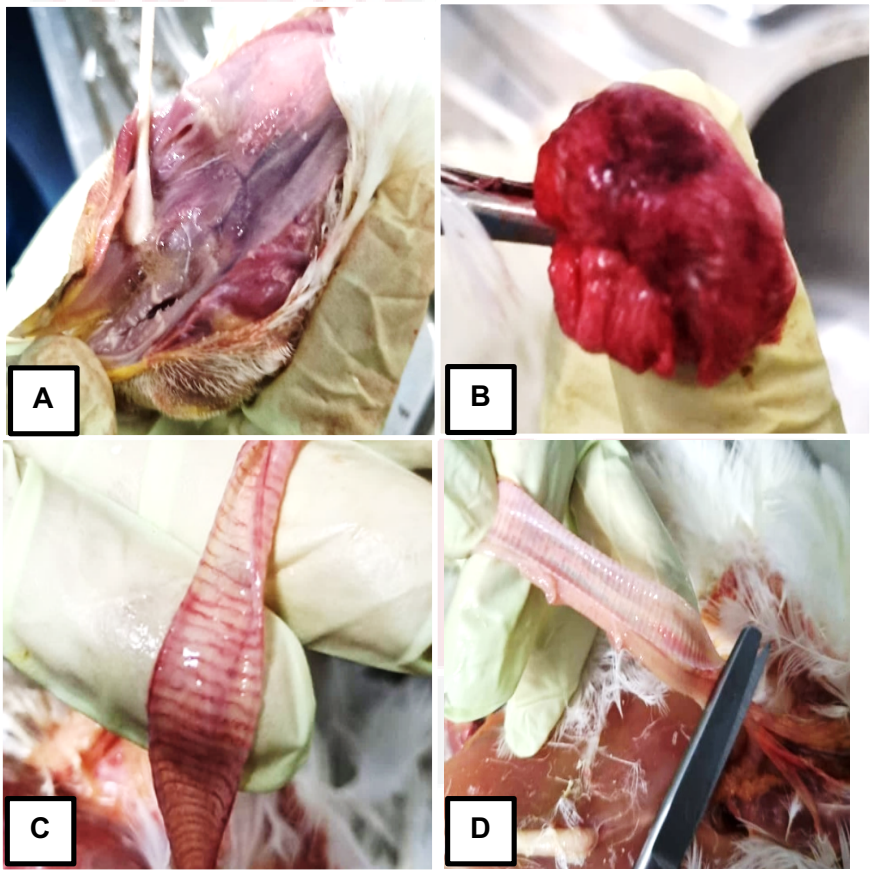

Figure 4.2 : *Death chickens from group 1(control group) showed post-mortem lesions such as A) mucus secretion in the respiratory tract, B) haemorrhage on the lungs, C) haemorrhage on the trachea, D) mucus secretion in the trachea.*

5.0 Discussion

The protective efficacy of the genotype-matched vaccine mIBS025 administered via single ED vaccination and double DW vaccination was studied in chickens challenged with velogenic genotype VII.2 NDV strain UPM008/2021 at 35 days old. The vaccine's protective efficacy was determined by determining the morbidity and mortality of the chickens after they were challenged with the virus. As expected, all of the chickens in the control groups infected with the virus resulted in 100% mortality after 12 days post-challenge. The chickens have no antibodies and are, therefore completely susceptible to the challenged virus. The infected chickens developed acute morbidity signs such as swollen eyes, nasal discharge, ruffled feathers, greenish white diarrhoea and depression after 4 days post-challenge.

On the other hand, chickens from the vaccinated groups via single ED vaccination and double DW vaccination were 100% protected. The vaccine administered via both routes is able to offer full protection against morbidity and mortality after post-challenged with velogenic NDV genotype VII. However, according to the study by Mahamud *et al.,* (2022), the single ED vaccination group was able to offer full protection against morbidity and mortality, but the single DW vaccination groups only showed 30% protection against morbidity at day 7 post-challenge (pc) and a 50% protection against mortality at day 11 pc. This indicates that increasing the frequency of vaccine administration can boost the immune response and increase the level of protection against the disease. Nasser *et al.* (2010) conducted a study in which groups of chickens were vaccinated via intraocular and drinking water routes at 2, 5,

and 9 weeks of age. In the results, both groups vaccination titers fell below the $log2^3$ protection titer after the first vaccination. Then, after the second and third boosters administered, the vaccination titer returned to $log2^3$, indicating that increasing the frequency of vaccine administration was able to increase the level of immune body protection. Both vaccinated groups were also challenged with virulent NDV at weeks 8 and 12, and all the birds were protected from clinical signs and mortality. However, increasing the frequency of vaccine administration does not always imply that the immune response of the body will improve, as the time interval between each vaccination booster is also important. According to Allan (1978), if the time interval between primary and secondary vaccination is less than 21 days, the first vaccination's antibodies are likely to interfere with the multiplication of the second dose of the vaccine virus leading to poor immunity development for the booster vaccine.

The DW route and ED route were used in the study because they are the most commonly used vaccination routes in the industry and mimic a natural infection of ND (Okwor *et al.,* 2013). When compared to DW vaccination, ED vaccination is more accurate, uniform, and provides better protection, but the vaccination programme is more labour intensive because it requires labour to catch the chickens. Meanwhile, DW vaccination is easier to administer, requires less labour, and produces less stress on the birds (Getabalew *et al.,* 2019). However, the downside is that the vaccine might not be uniformly distributed (Kouwenhoven, 1993).

ED vaccination also induces a significantly higher level of circulating antibodies in the chickens compared to DW vaccination (Alder and Spradbrow, 2001). The ED

vaccination produced a higher antibody titer (8log2–9log2) than the DW vaccination, which produced antibody titer of 3log2–6log2 (Okwor *et al.,* 2013). ED vaccination has higher vaccine efficacy because it can produce a higher level of circulating antibodies due to the presence of the Harderian gland, which is located behind the eyes. In chickens, the Harderian gland is an important organ in the development of immune responses (Chowdhury *et al.,* 1982). The Harderian glands are able to produce noticeably more plasma cells, which in turn produce the vital local and humoral antibodies to protect the body against the virus (Jayawardane and Spradbrow, 1995). Through the ED vaccination, large quantities of the vaccine viruses move into the Harderian gland able to escape the chickens' humoral and phagocytic antiviral systems, which results in better immune responses (Okwor *et al.,* 2013). Meanwhile, the fact that DW vaccination has lower vaccine efficacy could be due to some vaccine viruses being destroyed by the gastric secretions, resulting in poor immunity development (Okwor *et al.,* 2013).

A post mortem examination was performed to examine the development of gross lesion by the velogenic genotype VII.2 NDV strain. Among the gross lesions that were observed are mucus secretion in the respiratory tract and trachea and haemorrhage on the lungs and trachea. The respiratory tract was the most severely affected and these findings may imply that the chicken died as a result of respiratory failure. The NDV enter the body through the ocular route and replicate in the upper respiratory tracts. After infection, the virus spreads quickly through the blood, resulting in secondary viraemia. Clinical signs such as respiratory distress and dyspnoea would result from the congestion of the lungs and respiratory tract, as well as damage to the respiratory centre

in the brain. This results in breathing difficulties that reduce oxygen transport, and lead to hypoxia in the surrounding tissues. The tissue and cells would gradually necrose, including vital organs such as the brain, resulting in respiratory and cardiovascular system failure and death (Brown *et al.,* 1999)

6.0 Conclusion

Based on the results of morbidity and mortality of the chickens, genotype-matched NDV vaccine mIBS025 following single ED vaccination and double DW vaccination provide the same level of protection against ND. Since both routes provide the same level of protection, the suggested route of choice would be double DW vaccination due to the benefits of ease of administration, requiring less labour, and it causing less stress on the chickens. This allows large-scale farm to vaccinate their chickens more effectively.

7.0 Recommendations

In the future, similar studies should include an oropharyngeal swab to determine the efficacy of vaccines administered via different routes in preventing virus shedding. Morbidity and mortality rates in the current study can only tell us how well the vaccine protects chickens from clinical signs but not from virus shedding. Vaccinated chickens may appear healthy, but they can still shed the virus and cause an outbreak in unprotected chickens.

Furthermore, the subject of the study can be changed from SPF chickens to broilers to test the vaccine's efficacy. This is because the broiler's maternal antibodies may interfere with the vaccine strain, resulting in a lower level of immunity. This could imply that maybe ED vaccination might be a better vaccination route for the broilers.

In addition, the efficacy of the genotype-matched vaccine mISB025 can be tested against other NDV genotypes. There are 21 NDV genotypes classified in Class II categories, and only genotype VII.2 was challenged on the chicken to study the efficacy of the vaccine.

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