UNIVERSITI PUTRA MALAYSIA

IMMUNOGENICITY OF GENOTYPE-MATCHED NEWCASTLE DISEASE VIRUS VACCINE FOLLOWING SINGLE EYEDROP VACCINATION IN SPECIFIC-PATHOGEN-FREE CHICKENS

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It is hereby certified that we have read this project paper entitled "Immunogenicity of Genotype-Matched Newcastle Disease Virus Vaccine Following Single Eyedrop Vaccination in Specific-Pathogen Free Chickens", by Ong Yu Yen and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD4999 - Final Year Project.

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LIST OF ABBREVIATIONS

AOAV-1	Avian orthoavulavirus serotype-1
ARF	Animal Research Facility
AUP	Animal utilization protocol
dpv	Days post-vaccination
ECE	Embryonated chicken egg
ED	Eyedrop
EID_{50}	Embryo infectious dose 50
F	Fusion
HI	Haemagglutination inhibition
HN	Hemagglutinin-neuraminidase
IACUC	Institutional Animal Care and Use Committee
L	Large
M	Matrix
MDA	Maternally-derived antibodies
ND	Newcastle disease
nd	Not detected
NDV	Newcastle disease virus

NP	Nucleocapsid
OIE	World Organisation for Animal Health
P	Phosphoprotein
PBS	Phosphate-buffered saline
RBC	Red blood cell
SD	Standard deviation
SPF	Specific-pathogen-free
UPM	Universiti Putra Malaysia
˚C	Degree Celsius
HAU	Haemagglutination unit
mL	Milliliter
µL	Microliter
%	Percentage

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD4999 - Projek Ilmiah Tahun Akhir.

IMUNOGENISITI VAKSIN PADANAN-GENOTIP VIRUS PENYAKIT NEWCASTLE SELEPAS VAKSINASI TUNGGAL MELALUI TITISAN MATA PADA AYAM BEBAS PATOGEN SPESIFIK

Oleh

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2022

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Wabak penyakit Newcastle (ND) masih dilaporkan walaupun pelaksanaan program vaksinasi secara intensif, mengakibatkan kerugian besar di seluruh dunia. Kebanyakan vaksin ND komersial tergolong dalam genotip I atau II, manakala strain virus yang

menyebabkan wabak adalah genotip VII. Vaksin padanan-genotip ND, mIBS025 telah dihasilkan baru-baru ini. Kajian ini bertujuan untuk menyiasat imunogenisiti mIBS025 berikutan vaksinasi tunggal melalui titisan mata (ED) dengan dos virus vaksin yang berbeza pada ayam bebas patogen spesifik. 75 ekor ayam SPF berumur 14 hari telah dibahagikan kepada lima kumpulan. Kumpulan 1 telah diberikan dengan *phosphate-buffered saline* sebagai kumpulan kawalan yang tidak divaksin. Kumpulan 2 hingga 5 masing-masing telah divaksin dengan $10^{4.5}$, 10^{5}, $10^{5.5}$, dan 10^{6} dos berjangkit embrio 50 (EID_{50}) mIBS025 melalui ED. Sampel darah ayam telah dikumpul pada 7, 14 dan 21 hari pasca vaksinasi (dpv) untuk menentukan titer antibodi ND melalui ujian perencatan hemaglutinasi. Semua ayam telah dipantau setiap hari untuk tanda-tanda klinikal pasca vaksinasi selama tiga minggu. Pada 7 dpv, titer antibodi kumpulan $10^{5.5}$ EID_{50} adalah lebih tinggi (6.73 $\log_2$) secara signifikan ($p < 0.05$) daripada $10^{4.5}$ EID_{50} (5.50 $\log_2$). Pada 21 dpv, titer antibodi kumpulan 10^{6} EID_{50} adalah lebih tinggi (9.09 $\log_2$) secara signifikan ($p < 0.05$) daripada 10^{5} EID_{50} (8.12 $\log_2$). Semua ayam tidak menunjukkan kesan sampingan pasca vaksinasi. $10^{4.5}$ EID_{50} dianggap sebagai dos optimum mIBS025 kerana ia telah mengaruhkan titer pelindung ($\geq$ 5 $\log_2$) terhadap virus ND genotip VII pada 7 dpv. Oleh itu, dos dengan keperluan virus vaksin yang lebih rendah telah dipilih. Kajian ini akan memberi maklumat tentang imunogenisiti mIBS025 bagi kajian yang akan datang.

Kata Kunci: penyakit Newcastle; poltri; titisan mata; vaksin padanan-genotip ND; virus ND genotip VII

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD4999 - Final Year Project.

IMMUNOGENICITY OF GENOTYPE-MATCHED NEWCASTLE DISEASE VIRUS VACCINE FOLLOWING SINGLE EYEDROP VACCINATION IN SPECIFIC-PATHOGEN-FREE CHICKENS

By

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2022

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Newcastle disease (ND) outbreaks are still being reported despite intensive vaccination programme, causing substantial losses worldwide. Most commercial ND vaccines belong to genotype I or II, while the field strain responsible for outbreaks is genotype VII. A genotype-matched ND vaccine, mIBS025 has been developed

recently. This study aimed to investigate the immunogenicity of mIBS025 following single eyedrop (ED) vaccination with different doses of vaccine virus in specific-pathogen-free (SPF) chickens. 75 SPF chickens of 14-day-old were divided equally into five groups. Group 1 was administered with phosphate-buffered saline as unvaccinated control group. Group 2 to 5 were vaccinated with mIBS025 through ED with $10^{4.5}$, 10^{5}, $10^{5.5}$, and 10^{6} embryo infectious dose 50 (EID_{50}), respectively. Blood samples were collected from all birds at 7, 14, and 21 days post-vaccination (dpv) to determine ND antibody titers through haemagglutination inhibition test. All birds were monitored daily for post-vaccination clinical signs for three weeks. At 7 dpv, antibody titers of $10^{5.5}$ EID_{50} group is significantly ($p < 0.05$) higher (6.73 $\log_2$) than $10^{4.5}$ EID_{50} (5.50 $\log_2$). At 21 dpv, antibody titers of 10^{6} EID_{50} group is significantly ($p < 0.05$) higher (9.09 $\log_2$) than 10^{5} EID_{50} (8.12 $\log_2$). All birds did not exhibit post-vaccination side effects. $10^{4.5}$ EID_{50} was regarded as the optimal dose of mIBS025 as it has induced protective titers ($\geq$ 5 $\log_2$) against genotype VII ND virus at 7 dpv. Therefore, dose with a lower requirement of vaccine virus was chosen. This study provides information on immunogenicity of mIBS025 for future study.

Keywords: eyedrop; genotype-matched ND vaccine; genotype VII NDV; Newcastle disease; poultry

1.0 INTRODUCTION

1.1 Background

Newcastle disease (ND) is a highly contagious disease affecting a wide range of domestic and wild avian species. ND is caused by the avian orthoavulavirus serotype-1 (AOAV-1), which belongs to the order *Mononegavirales*, subfamily *Avulavirinae*, family *Paramyxoviridae* and genus *Orthoavulavirus* (ICTV, 2019).

ND is characterized by respiratory, neurological, gastrointestinal, and reproductive signs (Dimitrov *et al.*, 2017) such as dyspnoea, nasal and eye discharges, tremors, paralysis, neck twisting, greenish watery diarrhoea, decreased egg production, weakness, prostration, weight loss, and followed by death (Bello *et al.*, 2018; Pazhanivel *et al.*, 2002). The disease has had a devastating economic impact on poultry industry around the world, resulting in significant mortality and morbidity rates, as well as production-related losses (Bello *et al.*, 2018). Therefore, ND is listed in the World Organisation for Animal Health (OIE) Terrestrial Animal Health Code as a disease that must be reported immediately to the OIE upon detection (OIE, 2012).

Vaccination is practiced extensively in the poultry industry to prevent and control the spread of the disease (Zhao *et al.*, 2014). However, ND outbreaks are still being reported even among the vaccinated flocks, causing substantial losses. This problem arose probably due to genetic mismatch between the current ND vaccine strains and the circulating field strains (Fawzy *et al.*, 2020). All ND vaccines that are available commercially, namely B1, LaSota, and V4, are categorized as genotype I or II ND

virus (NDV), while the field strain responsible for disease outbreaks, particularly in Asia, belong to genotype VII NDV. Recent studies have indicated that genotype VII NDV has a significant impact on poultry production in Malaysia (Mahamud *et al.*, 2022).

Based on the study done by Mahamud *et al.* (2022), the author has employed reverse genetic technology to develop a genotype-matched live attenuated ND vaccine, mIBS025 by rationally attenuate the IBS025/13 at a motif in the fusion (F) gene, which is the most important determinant of NDV virulence (de Leeuw *et al.*, 2005; Kim *et al.*, 2013; Bello *et al.*, 2020b). It has been demonstrated that the genotype-matched ND vaccine, mIBS025 developed based on the field strain genotype VII NDV are able to provide better protective immunity to the chickens as compared to genotype I and II vaccines, particularly in terms of reducing the viral load and post-challenge virus shedding period. According to the study, a single vaccination via eyedrop (ED) with 10^6 embryo infectious dose 50 (EID_{50}) mIBS025 is able to induce high haemagglutination inhibition (HI) antibody titers at 7 log_2 that confer full protection (100%) against challenge with velogenic genotype VII in SPF chickens. However, the immunogenicity of the genotype-matched NDV vaccine, mIBS025 vaccine following ED vaccination with different doses of the vaccine virus is not known.

Therefore, the study of the immunogenicity of the genotype-matched NDV vaccine, mIBS025 following single ED vaccination with different doses of the vaccine virus in SPF chickens is warranted. The results of this study help to determine the optimal dose of genotype-matched NDV vaccine, mIBS025 to vaccinate the chickens via ED.

1.2 Objectives and Justification

This study was conducted with detailed objectives as below:

1. To determine the immunogenicity of the genotype-matched NDV vaccine, mIBS025 following single eyedrop vaccination with different doses of the vaccine virus in SPF chickens.
2. To determine the optimal dose of genotype-matched NDV vaccine, mIBS025 in inducing HI antibody titer in SPF chickens.
3. To evaluate any side effects of genotype-matched NDV vaccine, mIBS025 at different doses in SPF chickens.

The immunogenicity of different doses of mIBS025 and its optimal dose in inducing HI antibody titer following single eyedrop vaccination is not known. The findings of this study help to determine the optimal dose of mIBS025 to vaccinate the SPF chickens via eyedrop, and also provide insight on immunogenicity of mIBS025 for future vaccine study.

1.3 Hypotheses

The hypotheses that were tested in this study are:

H_0 = SPF chickens vaccinated with different doses of genotype-matched NDV vaccine, mIBS025 via eyedrop do not induce different immunogenicity.

H_A = SPF chickens vaccinated with different doses of genotype-matched NDV vaccine, mIBS025 via eyedrop do induce different immunogenicity.

2.0 LITERATURE REVIEW

2.1 Newcastle Disease

Newcastle disease (ND) is a highly contagious avian disease caused by the avian orthoavulavirus serotype-1 (AOAV-1) (ICTV, 2019). A wide range of domestic and wild avian species are susceptible to this viral disease, which has disastrous economic effects on the poultry industry (Alexander, 2001).

ND is characterized by respiratory, neurological, gastrointestinal, and reproductive signs (Dimitrov *et al.*, 2017). Respiratory signs include dyspnoea, gasping, coughing, nasal and eye discharges, and edema and cyanosis of comb and wattle; neurological signs include opisthotonos, tremors, neck twisting, and paralysis; gastrointestinal sign includes greenish watery diarrhoea; reproductive sign includes decreased egg production; while other general signs include weakness, prostration, ruffled feathers, loss of appetite, weight loss, and followed by death (Bello *et al.*, 2018; Pazhanivel *et al.*, 2002). Petechial haemorrhages, ulcers with elevated borders on the proventriculus mucosa, pneumonic lungs, and haemorrhages in the trachea, air sacs, brain, and spleen are among the gross abnormalities of ND (Pazhanivel *et al.*, 2002).

Transmission of ND to healthy birds can occur through direct or indirect contact. Direct contact transmission occurs through ingestion or inhalation of secretions from the respiratory tract, mouth, cloaca or eyes of infected animals. Indirect contact transmission occurs through contaminated feed, equipment, transport, clothing, and footwear (Dzogbema *et al.*, 2021).

In 1926, the first ND outbreak has occurred in Java, Indonesia (Kraneveld, 1926) and in Newcastle upon Tyne, Great Britain (Doyle, 1927). ND is distributed worldwide and is enzootic in Asia, Africa, Middle East, and some countries in Central and South America (Dzogbema *et al.*, 2021). The disease has causes enormous impacts on poultry farming with high mortality and morbidity rate and production-related losses (Bello *et al.*, 2018). Therefore, ND is listed in the World Organisation for Animal Health (OIE) Terrestrial Animal Health Code as a disease that must be reported immediately to the OIE upon detection (OIE, 2012).

2.2 Aetiology

The causative agent of ND is avian orthoavulavirus serotype-1 (AOAV-1), which belongs to the order *Mononegavirales*, subfamily *Avulavirinae*, family *Paramyxoviridae* and genus *Orthoavulavirus* (ICTV, 2019). The ND virus (NDV), synonymous with AOAV-1, is a single-stranded non-segmented negative-sense enveloped ribonucleic acid (RNA) virus with about 15.2 kilobase pairs genome (Aljumaili *et al.*, 2017).

NDV genome encodes for six structural proteins, which are hemagglutinin-neuraminidase (HN) protein, fusion (F) protein, matrix (M) protein, nucleocapsid (NP) protein, phosphoprotein (P), and large (L) protein, as well as two non-structural proteins known as V and W (Murulitharan *et al.*, 2013).

AOAV-1 are genetically classified into class I and class II. Class I isolates are grouped into only one genotype, whereas class II isolates are subdivided into genotypes I to XVIII (Dimitrov *et al.*, 2017).

NDV strains can be categorized into five pathotypes based on the severity of clinical signs in infected birds: viscerotropic velogenic (Doyle's form), neurotropic velogenic (Beach's form), mesogenic (Beaudette's form), lentogenic (Hitchner's form), and aymptomatic (Alexander and Senne, 2008). Viscerotropic velogenic is characterized by an acute lethal infection in all ages with haemorrhagic intestinal lesions; neurotropic velogenic characterized by an acute lethal infection in all ages with respiratory and neurological signs; mesogenic characterized by respiratory and neurological signs with mortality usually in young birds; lentogenic characterized by mild or inapparent respiratory infections (commonly used as vaccines); and asymptomatic characterized by avirulent enteric infections without causing obvious disease (Beard and Hanson, 1984).

2.3 Current Scenario in Malaysia

In Malaysia, the first report of ND case among the poultry flocks was in 1934 in Parit Buntar, Perak (Anon, 1934). It has continuously cause major disease outbreaks and remain a constant threat to both commercial and backyard poultry operations nationwide (Leow *et al.*, 2011).

Vaccination is practiced extensively in the poultry industry to prevent and control the spread of the disease (Zhao *et al.*, 2014). However, ND outbreaks are still being reported even among the vaccinated flocks, causing substantial losses.

Based on the molecular epidemiology study done by Berhanu *et al.* (2010), it has indicated that genotype VII NDV is the predominant virus strain that are circulating in Malaysia currently. According to the unpublished data by Department of Veterinary Services, Ministry of Agriculture and Malaysia, the index for reported genotype VII NDV outbreaks in Malaysia in 2009 was 5, while 2010 was 75. In 2011, the outbreaks become more serious with an index of 153 (Roohani *et al.*, 2015).

This problem arised probably due to genetic mismatch between the current NDV vaccine strains and the circulating field strains (Fawzy *et al.*, 2020). All ND vaccines that are available commercially, namely B1, LaSota, and V4, are categorized as genotype I or II NDV, which are different from the field strain genotype VII NDV that is responsible for disease outbreaks in Malaysia (Mahamud *et al.*, 2022).

2.4 Control of Disease through Vaccination

As there is no cure for ND (Dzogbema *et al.*, 2021), the general approach to the control of ND is through vaccination, which would elicit an immunological response against the infection (Getabalew *et al.*, 2019). The three main goals of vaccination against ND are i) prevent clinical disease or reduce its severity; ii) decrease virulent virus shedding; and iii) increase the infectious dose of the challenge virus (Kapczynski *et al.*, 2013).

Types of vaccines used to control ND can be classified into two, which are conventional vaccines and recombinant vaccines (Bello *et al.*, 2018).

2.4.1 Conventional Vaccines

Examples of conventional vaccines are live attenuated vaccines and inactivated vaccines (Senne *et al.*, 2004).

NDV strains used to produce commercial live attenuated vaccines are divided into two groups, which are lentogenic (LaSota, Hitchner-B1, V4, I2, F, NDW) and mesogenic (Roakin, Mukteswar, Komarov) (OIE, 2018). Among the lentogenic vaccine strains, LaSota has shown superior immunogenicity by inducing high levels of neutralizing antibodies in naive chickens as compared to other strains (Meulemans, 1988). Therefore, LaSota strain is widely used around the world especially in countries where ND is endemic (Diel *et al.*, 2012). The highly attenuated Hitchner-B1 strain is not as immunogenic as LaSota, but is more suitable to be used in young chickens as it does not cause post-vaccination respiratory reactions (Bello *et al.*, 2018). For V4 and I2 strains, they are renowned for their thermostable feature which make them tolerate elevated temperature in area where refrigeration facilities are limited (Bensink and Spradbrow, 1999). Among the mesogenic vaccine strains, Komorov and Mukteswar are usually used as booster vaccine strains following priming with lentogenic isolates (Senne *et al.*, 2004).

All live attenuated vaccines are able to elicit robust systemic and mucosal immunity capable of protecting the chickens from clinical disease (Bello *et al.*, 2018).

Furthermore, mass application through drinking water or spray can be employed to administer vaccines, hence reducing the time and labour needed (Geus *et al.*, 2012). Moreover, the spread of vaccine virus from well-vaccinated chickens to suboptimally vaccinated chickens around them is able to contribute to the overall herd immunity (Thornton *et al.*, 1980).

However, the virulent virus is still persisted in the environment due to the continuous shedding of challenged virus via the cloacal and oropharyngeal routes (Bello *et al.*, 2018). On top of that, vaccinated chickens may develop clinical illness as the live virus has the potential to revert back to its virulent state. Additionally, some vaccine strains may cause young chickens to suffer post-vaccination respiratory reactions, which may predispose the chickens for developing secondary bacterial infections (Winterfield *et al.*, 1980). What's more, the commercially available live attenuated vaccines are of genotype I or II strains, which are distinct from the currently prevalent field strain genotype VII that is responsible for disease outbreaks in Malaysia (Mahamud *et al.*, 2022).

Another type of conventional vaccines is the inactivated vaccines. Inactivated vaccines differ from live attenuated vaccines in that they cannot replicate in the host (Getabalew *et al.*, 2019). To produce the vaccines, the NDV strain of interest is grown to high titers in eggs, which then its infective allantoic fluid is treated with inactivating agent (Getabalew *et al.*, 2019), such as binary ethylenimine (BEI) and formaldehyde (Razmaraii *et al.*, 2012). Care should be taken during the inactivation process by sparing the immunogenic epitopes of viral surface glycoproteins HN and F, as they are the major determinants of neutralizing antibodies (Bello *et al.*, 2018).

Since the virus has been inactivated and no longer capable of replication, there is no risk that it will revert back to its virulent form. Inactivated vaccines are usually given after initial priming with live attenuated vaccines in order to achieve optimal immune responses (Zhai *et al.*, 2011). The vaccines are prepared in emulsion of mineral oil as adjuvant to make the inactivated virus more immunogenic (Aljumaili *et al.*, 2020).

However, the vaccine has to be administered through parenteral route (intramuscularly or subcutaneously) to every chickens individually because the virus is not able to multiply and disseminate horizontally among the vaccinated chickens (Getabalew *et al.*, 2019). This requirement of individual handling is very time-consuming and labour intensive, making the vaccination process expensive and inefficient. Furthermore, adjuvants used in inactivated vaccines may cause some undesirable effects in the vaccinated chickens. It is well documented that some mineral oils can induce adverse tissue reactions in chickens. Some may also contain carcinogenic components that pose a higher likelihood of poultry meat consumers for developing cancer (Aljumaili *et al.*, 2020). Moreover, withdrawal period is needed before the chickens vaccinated with inactivated vaccines can be consumed by human (Kapczynski *et al.*, 2013). Last but not least, inactivated vaccines often fail to induce strong mucosal or cell-mediated immune response (Zhai *et al.*, 2011).

2.4.2 Recombinant Vaccines

One of the downsides associated with the conventional genotype I and II vaccines is that even if clinical protection is attained, they are unable to inhibit the shedding of the heterologous virulent NDV (Bello *et al.*, 2018). According to Choi *et al.* (2013b),

homologous vaccines that are genetically closer to the challenge strains, also known as genotype-matched vaccines, are more effective than heterologous vaccines at providing protection to the chickens. Hence, focus is currently on the development of genotype-matched vaccines for improved control of ND.

In recent years, a novel technique called reverse genetics has been employed to produce reverse genetic-based genotype-matched live attenuated ND vaccines. Reverse genetics is the recovery of a recombinant virus from its cloned complementary deoxyribonucleic acid (cDNA) (Pfaller *et al.*, 2015). By using reverse genetics technology, Hu *et al.* (2011) and Xiao *et al.* (2012) genetically altered the F cleavage site of a highly virulent genotype VII NDV and demonstrated its complete loss of virulence and ability to evoke an excellent protective immunity against clinical disease and reduce virus shedding significantly after exposure to a highly virulent wild type genotype VII NDV isolate.

Therefore, reverse genetics is an appealing strategy to rapidly generate genetically stable genotype-matched live attenuated ND vaccines that are homologous with the prevalent NDV strains with guaranteed protective efficacy (Bello *et al.*, 2018).

2.5 Development of Genotype-matched Vaccine in Malaysia

Based on the study done by Mahamud *et al.* (2022), the author has selected a virulent naturally detected recombinant NDV isolate, IBS025/13 as the parental virus for genotype-matched vaccine development. IBS025/13 was among the genotype VII NDV strains that were isolated from vaccinated poultry farms in Malaysia between

2004 – 2013. It appears to be a naturally occurring recombinant strain of NDV derived from the vaccination and field strains (Bello *et al.*, 2020).

IBS025/13 was selected as a candidate due to its high tissue tropism in both the respiratory and gastrointestinal systems (Bello *et al.*, 2018). Besides, the entire viral NP protein and approximately 67% of its P genes were those found in genotype II. Additionally, the HN, F, M, and L proteins were all shown to be closely linked to those seen in genotype VII isolates (Satharasinghe *et al.*, 2016). On top of that, the isolate's genomic length is 15186 base pairs, which is consistent with NDV genotypes detected before the 1960s. Importantly, the virus is highly virulent and multiplies rapidly in chicken embryonated eggs. The virus's distinct features make it an ideal candidate for vaccine development.

In that study, the author has employed reverse genetic technology to rationally attenuate the IBS025/13 at a motif in the F gene, which is the most important determinant of NDV virulence (de Leeuw *et al.*, 2005; Kim *et al.*, 2013; Bello *et al.*, 2020b). The F cleavage site was modified from virulent (polybasic) to avirulent (monobasic), as contained in all attenuated NDV strains (Gururaj *et al.*, 2014).

The attenuated virus was then tested in SPF chickens for its protective efficacy against challenge of genotype VII NDV. Pathogenicity tests revealed that the attenuated IBS025/13 was completely avirulent, as indicated by the OIE recommended pathogenicity testing indices. Moreover, the attenuated IBS025/13 was found to be genetically stable and replicated efficiently during passages in the respiratory organs of young chicks and in SPF chicken embryonated eggs, suggesting the virus stability. It has been demonstrated that the genotype-matched live attenuated ND vaccine,

mIBS025 developed based on the field strain genotype VII NDV are able to elicit strong humoral immunity to provide better protection against mortality and morbidity to the chickens as compared to genotype I and II vaccines, particularly in terms of reducing viral load and duration of virus shedding post-challenge.

Taken together, these findings suggest that mIBS025 is a feasible vaccine candidate for better genotype VII ND control in Malaysia.

2.6 Eyedrop Vaccine Administration

The most common routes of vaccination in poultry include drinking water, spray, eyedrop (ED), intramuscular or subcutaneous injection, wing web, and feed (Pattison *et al.*, 2008). Among these routes, the most effective means of administering live lentogenic vaccines to chickens is probably the ED, as each chicken will be handled individually and received a full dose of vaccine. Therefore, antibody titers obtained are usually uniform throughout the flocks (Getabalew *et al.*, 2019).

Through ED administration, the vaccine is passes to the Harderian gland located posterior to the eyeball where its secretory duct opens onto the surface of the nictitating membrane. It serves as a key component of the head associated lymphoid tissues (HALTs) in chickens, containing secretory bodies of antibodies and other immune cells (Payne, 1994), facilitating both local and humoral immunity in chickens (Cargill, 1999).

With the presence of abundant plasma cells (Bang and Bang, 1968; Wight *et al.*, 1971), surface immunoglobulin-bearing cells (Albini and Wick, 1973), and some antibody-

producing cells (Mueller *et al.*, 1971) in the gland, it is most probably significant in the local immunity of the upper respiratory tract and eyes. Research done by Sundick *et al.* (1973) has also revealed that the Harderian gland's lymphoid cells are mostly bursa-dependent, implying that the gland is involved in humoral immune responses.

However, this administration route is very time-consuming and labour intensive, as it requires individual handling of chickens (Pattison *et al.*, 2008).

2.7 Haemagglutination Inhibition Test

There are several laboratory tests for NDV, such as haemagglutination inhibition (HI) test, enzyme-linked immunosorbent assay (ELISA), and reverse-transcription polymerase chain reaction (RT-PCR) (Dzogbema *et al.*, 2021). Among these tests, the most popular reference test to evaluate the humoral immune responses post-vaccination is the HI test (Choi *et al.*, 2013a) as it is a simple, rapid, and inexpensive serological test for NDV surveillance. This test is utilized to quantify specific antibody titers in chickens.

The principle behind HI test is that the HN protein of NDV possess haemagglutination activity and is able to attach to the receptor on the surface of red blood cells (RBCs), causing NDV to agglutinate RBCs (Saif, 2008). When serum sample containing antibodies are incubated with corresponding NDV strains as haemagglutination antigens, the antibodies will attach to the antigen sites on HN protein of NDV. This prevents the HN protein from attaching to the receptor site on RBCs. As a result, the virus-RBCs haemagglutination reaction is blocked. HI titers were recorded as the

reciprocal of the highest serum dilution that completely inhibited the agglutination of RBCs by the HA antigens (Getabalew *et al.*, 2019).

3.0 MATERIALS AND METHODS

3.1 Ethical Clearance

This study was conducted under the supervision of the institution veterinarian in accordance with the Animal Welfare Act 2015, Universiti Putra Malaysia (UPM) policy and Code of Practice for the Care and Use of Animals for Scientific Purposes and Institutional Animal Care and Use Committee (IACUC) guidelines. The animal utilization protocol (AUP) in this study was approved by the IACUC with AUP number of UPM/IACUC/AUP-R040/2022 dated 10th August 2022.

3.2 Experimental Design

The study was conducted at the Animal Research Facility (ARF), Faculty of Veterinary Medicine, UPM.

75 fourteen-day-old specific-pathogen-free (SPF) chicks were divided equally into five treatment groups containing 15 birds per group. Group 1 was unvaccinated control group, while group 2, 3, 4, and 5 were vaccinated groups. The unvaccinated and vaccinated groups were housed separately in two different rooms.

Prior to vaccination at fourteen-day-old, 1 mL of blood samples were collected from 5 birds in each group via the jugular vein in order to check for pre-vaccination NDV antibody titers by using haemagglutination inhibition (HI) test to make sure that the birds are free of NDV antibody. At fourteen-day-old, all birds in group 1 were

administered with 0.1 mL of phosphate-buffered saline (PBS), while all birds in group 2, 3, 4, and 5 were administered with 0.1 mL of genotype-matched ND vaccine, mIBS025 through ED route with vaccination dose of $10^{4.5}$ embryo infectious dose 50 (EID_{50})/0.1 mL, 10^5 EID_{50}/0.1 mL, $10^{5.5}$ EID_{50}/0.1 mL, and 10^6 EID_{50}/0.1 mL, respectively.

All the birds were monitored daily post-vaccination for any clinical signs associated with the potential side effects of the vaccine for three weeks. 2 to 3 mL of blood samples were collected from all birds in each group via the jugular vein at 7, 14, and 21 days post-vaccination (dpv) to determine the immunogenicity of respective vaccination dose by using HI test.

3.3 Chickens

75 seven-day-old SPF embryonated chicken eggs were obtained from Malaysian Vaccines and Pharmaceuticals (MVP). The SPF eggs were incubated in egg incubator at 37°C.

The hatched birds were then transported to ARF. They were raised in clean stainless-steel bird cages in well-ventilated and thermoregulated rooms. The birds were fed with commercial chicken feed once per day, and ad libitum water was provided in bell drinkers. Bell drinkers were washed and water was changed on a daily basis, while the bedding and cages were cleaned once per week.

All birds in each group were wing tagged prior to the commencement of experiment to allow accurate monitoring of individual response to the genotype-matched ND vaccine.

3.4 Vaccination

The genotype VII ND vaccine virus, mIBS025 was used in this study. mIBS025 is a live attenuated vaccine virus with lentogenic pathotype. It is produced by attenuating the genotype VII NDV strain IBS025/13 through reverse genetic technology (Bello *et al.*, 2020a). In this study, the vaccine was given by using micropipette through ED route.

To prepare the virus titer needed for vaccination dose of 10^6 EID_{50}/0.1 mL and 10^5 EID_{50}/0.1 mL, 10^9 EID_{50}/mL of stock virus was diluted with PBS with dilution factor of 1:100 and 1:1000 respectively, to obtain 10^7 EID_{50}/mL and 10^6 EID_{50}/mL of stock virus. 0.1 mL of 10^7 EID_{50}/mL and 10^6 EID_{50}/mL of stock virus was taken and administered to the birds to achieve vaccination dose of 10^6 EID_{50}/0.1 mL and 10^5 EID_{50}/0.1 mL respectively.

To prepare the virus titer needed for vaccination dose of $10^{5.5}$ EID_{50}/0.1 mL and $10^{4.5}$ EID_{50}/0.1 mL, 10^9 EID_{50}/mL of stock virus was diluted with PBS with dilution factor of 1:3.16, to obtain $10^{8.5}$ EID_{50}/mL of stock virus. $10^{8.5}$ EID_{50}/mL of stock virus was further diluted with dilution factor of 1:100 and 1:1000 respectively, to obtain $10^{6.5}$ EID_{50}/mL and $10^{5.5}$ EID_{50}/mL of stock virus. 0.1 mL of $10^{6.5}$ EID_{50}/mL and $10^{5.5}$

EID_{50}/mL of stock virus was taken and administered to the birds to achieve vaccination dose of $10^{5.5}$ EID_{50}/0.1 mL and $10^{4.5}$ EID_{50}/0.1 mL respectively.

3.5 Sampling

For pre-vaccination screening at fourteen-day-old, 1 mL of blood samples were collected from 5 birds in each group. At 7, 14, and 21 dpv, 2 mL of blood samples were collected from all birds in each group. All the blood samples were collected by using 3 mL syringe and 25 gauge × 1 inch needle via the jugular vein. After the serum has separated, the serum was transferred to microcentrifuge tube by using micropipette. Each bird's tag number was labelled on respective blood collection syringe and microcentrifuge tube to allow monitoring of individual antibody titers.

3.6 Haemagglutination Inhibition Test

HI test is one of the most popular reference tests to evaluate the humoral immune responses post-vaccination for ND. The HI test was performed as described by the OIE Terrestrial Manual (OIE, 2018).

25 µL of PBS was dispensed into well 2 to 12 of all rows of V-bottomed 96-well plate. Next, 50 µL of serum was placed into the first well and two-fold dilutions of 25 µL of serum were made until well 11 of all rows. Then, 25 µL of 4 HAU antigen was added into all well except for the control well at column 12. The plate was then incubated at room temperature for 30 minutes. Next, 25 µL of 1% chicken red blood cells (cRBCs)

was added into all well, and the plate was then incubated at room temperature for another 30 minutes.

To read the results, the plate was tilted. Only those well in which the RBC stream at the same rate as the control well were considered to show inhibition. HI titers were recorded as the reciprocal of the highest serum dilution that completely inhibited the agglutination of RBC. HI antibody titers of 8 or below ($\leq 2^3$) were considered as negative for NDV antibody.

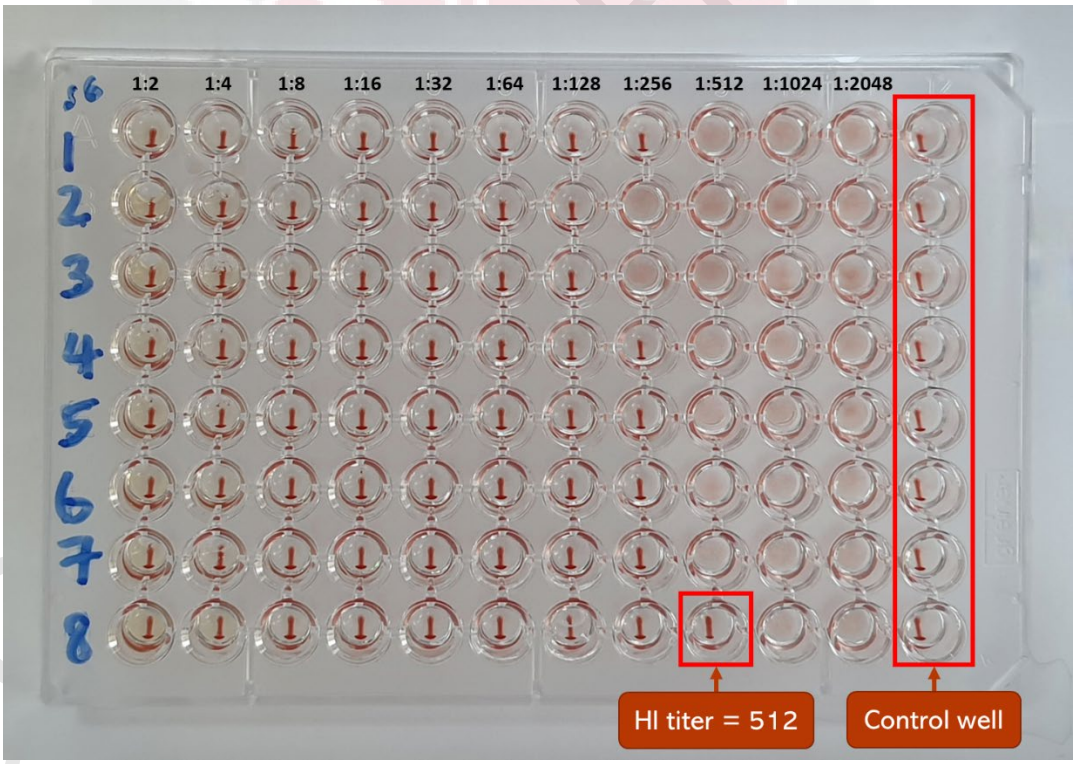

Figure 1: Haemagglutination inhibition test. For Row 8, the highest serum dilution that completely inhibited the agglutination of RBC was 1:512. Therefore, the HI titers for Row 8 was 512.

3.7 Statistical Analysis

IBM® SPSS® Statistics 23 was used to perform one-way analysis of variance (ANOVA) with Tukey post hoc test to analyze the significant differences between antibody titers of birds from different doses of vaccinated groups and unvaccinated control group. The results obtained were considered as statistically significant when the p-value was less than 0.05 ($p < 0.05$). The results are expressed as the geometric mean plus or minus (±) standard deviation (SD).

4.0 RESULTS

Results obtained are as shown in the line graph (Figure 2) and table (Table 1). The antibody titers are expressed in $\log_2$. Pre-vaccination screening confirmed that the fourteen-day-old chickens were free of NDV antibody. Chickens from unvaccinated control group remained negative for NDV antibody throughout the study. At 7 dpv, the antibody titers of chickens for all four vaccination doses range from 5.50 ± 1.22 to 6.73 ± 0.89 $\log_2$. The titers continue to increase and recorded a reading of 7.44 ± 0.94 to 7.98 ± 0.58 $\log_2$ at 14 dpv. At 21 dpv, the titers continue to rise ranging from 8.12 ± 0.66 to 9.09 ± 1.14 $\log_2$.

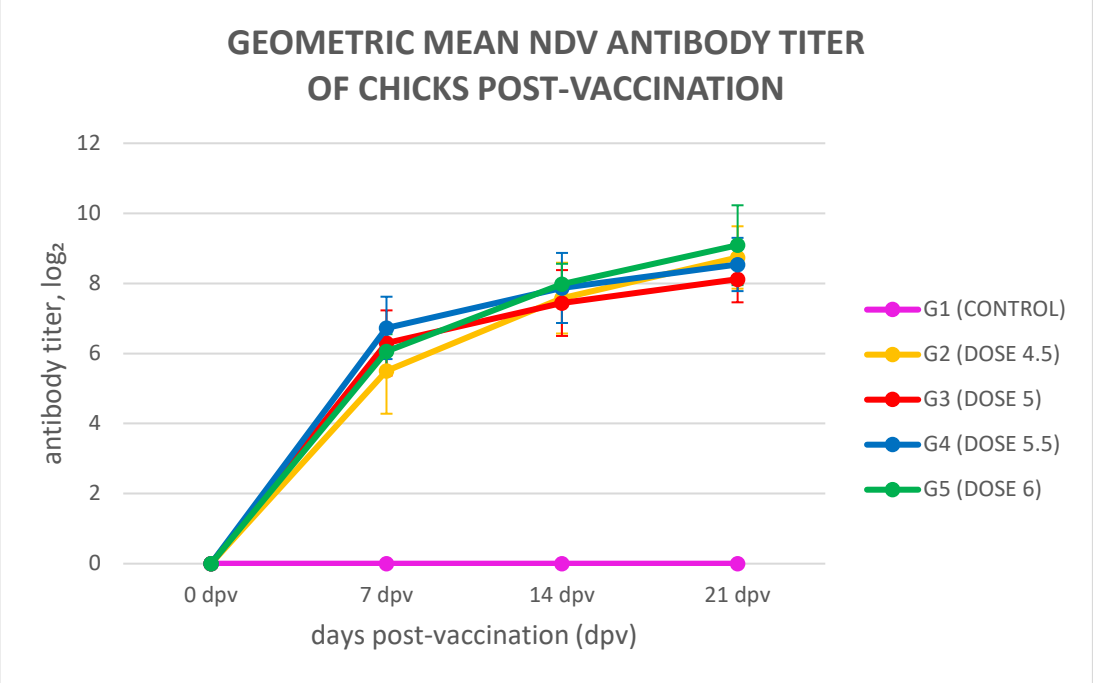

Figure 2: Geometric mean NDV HI antibody titer of SPF chickens post-vaccination.

Table 1: Geometric mean NDV HI antibody titer of SPF chickens post-vaccination.

Groups	Antibody titer, $\log_2$ (Geometric mean ± SD)			
	0 dpv	7 dpv	14 dpv	21 dpv
G1 - Control	nd[a]	ND[a]	ND[a]	ND[a]
G2 - $10^{4.5}$ EID_{50}	nd[a]	5.50 ± 1.22[b]	7.58 ± 1.01[b]	8.74 ± 0.89[bc]
G3 - 10^{5} EID_{50}	nd[a]	6.30 ± 0.93[bc]	7.44 ± 0.94[b]	8.12 ± 0.66[b]
G4 - $10^{5.5}$ EID_{50}	nd[a]	6.73 ± 0.89[c]	7.87 ± 1.00[b]	8.54 ± 0.76[bc]
G5 - 10^{6} EID_{50}	nd[a]	6.06 ± 0.49[bc]	7.98 ± 0.58[b]	9.09 ± 1.14[c]

Values with different superscript letters in a column are significantly different ($p < 0.05$).

Chickens with HI antibody titers of 8 or below ($\leq 2^3$) were considered as negative for NDV antibody.

dpv = Days post-vaccination

nd = Not detected

In general, all chickens from each vaccination doses showed steady increase in antibody titers. Besides, no post-vaccination side effects were observed throughout the study. All the chickens appeared healthy and active (Figure 3). In addition, no abnormal findings were observed during post-mortem examination (Figure 4).

Figure 3: Chickens post-vaccination.

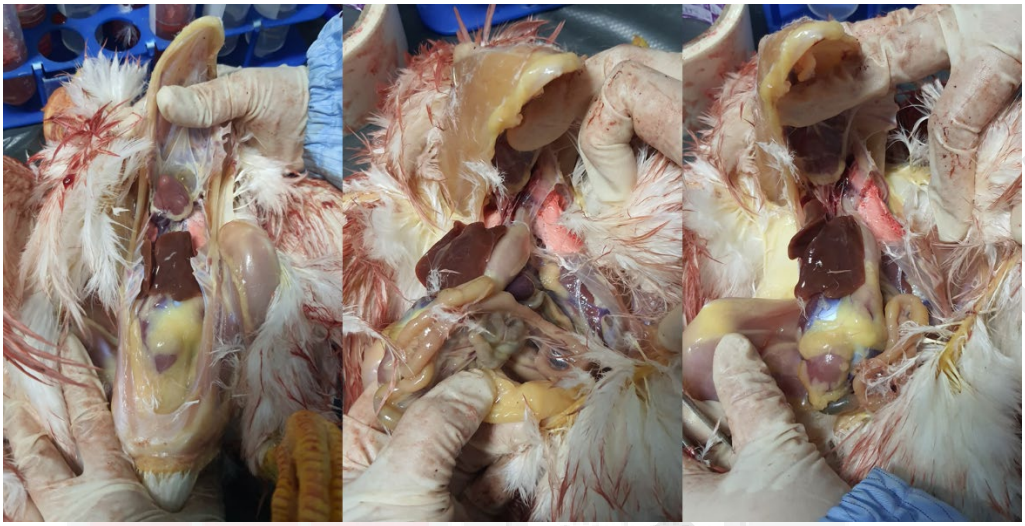

Figure 4: Post-mortem examination findings.

4.1 Determination of Optimal mIBS025 Vaccination Dose

The optimal dose of the vaccine was determined based on the antibody titers of the SPF chickens. At 7 dpv, $10^{5.5}$ EID_{50} with antibody titers of 6.73 ± 0.89 log_2 is significantly ($p < 0.05$) higher than $10^{4.5}$ EID_{50}, which is only 5.50 ± 1.22 log_2. However, there is no significant difference between 10^5 and 10^6 with $10^{5.5}$ EID_{50}. Hence, vaccination dose of 10^5, $10^{5.5}$, and 10^6 EID_{50} are recommended at 7 dpv.

At 14 dpv, there is no significant difference between all four doses. Thus, all four vaccination doses can be used at this time point.

At 21 dpv, 10^6 EID_{50} with antibody titers of 9.09 ± 1.14 log_2 is significantly ($p < 0.05$) higher than 10^5 EID_{50}, which is only 8.12 ± 0.66 log_2. However, there is no significant difference between $10^{4.5}$ and $10^{5.5}$ with 10^6 EID_{50}. Therefore, vaccination dose of $10^{4.5}$, $10^{5.5}$, and 10^6 EID_{50} are recommended at 21 dpv.

Overall, $10^{4.5}$ EID_{50} was regarded as the optimal dose of mIBS025 in inducing HI antibody titers in SPF chickens, this is because the antibody titers between $10^{4.5}$ and 10^{6} EID_{50} were not significantly different at 21 dpv. Furthermore, chickens vaccinated with $10^{4.5}$ EID_{50} mIBS025 has achieved protective antibody titers against genotype VII NDV at 7 dpv, which is more than 5 $\log_2$ (Bello *et al.*, 2020a). Therefore, dose with a lower requirement of vaccine virus was chosen.

5.0 DISCUSSION

ND is distributed worldwide and is enzootic in several countries (Dzogbema *et al.*, 2021), causing disastrous impacts on both commercial and backyard poultry operations (Leow *et al.*, 2011). For over 70 years, conventional ND vaccines such as LaSota and B1 have been used extensively in the poultry industry to protect the birds against overt clinical disease and control the spread of disease (OIE, 2018). However, continuous ND outbreaks are still being reported.

Several strategies have been implemented to control further ND outbreak, such as practice of strict and comprehensive biosecurity, and refinement of vaccination programme through hatchery vaccination and the use of recombinant vaccine, such as genotype-matched vaccine.

Genotype-matched vaccines are homologous vaccines that are genetically closer to the challenge strains. They are more effective than heterologous vaccines at providing protection to the birds (Choi *et al.*, 2013). Hence, focus is currently on the development of genotype-matched vaccines. In a previous study done by Bello *et al.* (2020), the author has employed reverse genetic technology to develop a genotype-matched live attenuated ND vaccine, mIBS025.

In another study done by Mahamud *et al.* (2022), mIBS025 developed based on the field strain genotype VII NDV has demonstrated ability to elicit strong humoral immunity, and reduce viral load and duration of virus shedding post-challenge as compared to genotype I and II ND vaccines.

In present study, the immunogenicity of mIBS025 in SPF chickens was evaluated in order to determine the optimal vaccination dose based on the antibody titers. From the results obtained, group vaccinated with a single ED vaccination with $10^{4.5}$ EID_{50} mIBS025 has achieved antibody titers of 8.74 ± 0.89 log_2 at 21 dpv. A previous study has shown that a single ED vaccination with 10^6 EID_{50} mIBS025 is able to induce high antibody titers at 7 log_2 that confer 100% protection against clinical disease and reduce virus shedding substantially as compared to LaSota vaccine (Mahamud *et al.*, 2022). This indicate that vaccination dose of $10^{4.5}$ EID_{50} is sufficient to provide full protection to the SPF chickens against challenge with velogenic genotype VII NDV in SPF chickens, without causing any post-vaccination side effects and post-mortem abnormalities, while requiring lower amount of vaccine virus to be used to produce the vaccine.

In this study, uniform antibody titers have been demonstrated throughout each vaccinated group. The uniformity was due to individual handling of the chickens, which allowed each chicken to receive a full dose of vaccine. However, this route of administration is very time-consuming and labour intensive, which indirectly increase the cost of production of the farm.

Several criteria of an ideal vaccine have been possessed by mIBS025. The first criterion is safe. All vaccinated chickens did not show any clinical signs associated with the potential side effects of the mIBS025 vaccine throughout the study. All chickens remained healthy and active post-vaccination. This is because the parental virus of mIBS025 was well-attenuated and was completely avirulent as revealed by the pathogenicity tests. The vaccine virus was also found to be genetically stable and

replicated efficiently during passages in the respiratory organs of young chicks and in SPF chicken embryonated eggs, suggesting its stability (Bello *et al.* 2020).

The second criterion is cost-effective. An ideal vaccine should be at an affordable price for the farmer, while remain effective in providing protection to the chickens. This criterion is well-demonstrated by mIBS025 vaccine, where vaccination dose with lower requirement of vaccine virus was sufficient to induce protective titers against genotype VII NDV in SPF chickens, therefore optimizing the cost of production. In addition, since mIBS025 are produced locally in Malaysia, the cost of vaccine will be cheaper as there are no foreign exchange issues (Alexander *et al.*, 2004). A more affordable vaccine means there will be more chickens to get vaccinated and protected from the disease, and would contribute to improved control of ND.

The third criterion is strong and fast onset of immunity. All chickens vaccinated with mIBS025 has achieved antibody titers of more than 5 $\log_2$, which is the protective titers against genotype VII NDV as early as 7 dpv (Bello *et al.*, 2020a). This indicate that the vaccine is able to provide early protection to the chickens, therefore minimizing the risk of infection.

The fourth criterion is ability to reduce virus shedding. According to a previous study done by Mahamud *et al.* (2022), mIBS025 is able to reduce viral load and duration of virus shedding post-challenge substantially as compared to genotype I and II ND vaccines, as the vaccine strain is homologous with the field challenge strain. As a result, replication of virulent NDV and shedding of virus into the environment could be blocked, hence reducing potential outbreaks among the non-protected in-contact chickens (Rehmani *et al.*, 2015).

In this study, SPF chickens were used to determine the immunogenicity of mIBS025 instead of commercial chickens. Consideration should be given to several factors in the field that would affect the vaccine's immunogenicity. One of the major factors would be maternally-derived antibodies (MDA) interference. Although MDA could provide early protection to young chicks from disease, but high level of MDA may interfere with vaccine-induced immune responses (Bertran *et al.*, 2018). However, other researchers have proved that vaccine virus introduced through ocular route is able to multiply on the respiratory epithelium without being neutralized by the circulating MDA (Beaudette and Bivins, 1953). Moreover, live ND vaccine introduced through ED route were found to be able to induce best response, due to its ability to induce development of local immunity (Dimitrov *et al.*, 2017). Therefore, if mIBS025 is to be administered through ED vaccination in the field setting, it is believed that similar immunogenicity as in this study could be induced.

The next factor that would affect the immunogenicity of vaccine is the chicken's immune competence. The presence of immunosuppressive diseases such as infectious bursal disease (IBD), infectious bronchitis (IB) and mycoplasmosis will cause weakened immune system (Dimitrov *et al.*, 2017). These immunosuppressed chickens are not capable of mounting strong immune response when vaccinated against NDV, leading to reduced protective efficacy.

Other field-associated factors unrelated to the vaccines include inappropriate vaccination techniques, not following recommended vaccination schedule, poor vaccine handling, untrained staff leading to faulty administration in chickens, lack of proper storage, use of expired vaccines, poor vaccine handling, etc (Sharif *et al.*, 2014).

All these factors should be taken into consideration when implementing a vaccination programme in order to ensure its success, hence a better control of disease and increased productivity of the farm.

6.0 CONCLUSION AND RECOMMENDATIONS

In conclusion, different immunogenicity has been induced in SPF chickens vaccinated with different doses of mIBS025 via single ED vaccination. Next, $10^{4.5}$ EID_{50} is the optimal dose of mIBS025 in inducing HI antibody titer in SPF chickens. Lastly, no side effects associated with different doses of mIBS025 were observed.

Current study is the initial step in the investigation of immunogenicity of mIBS025 vaccine. Therefore, the results obtained should be interpreted with caution as SPF chickens were used in this study to evaluate the immunogenicity of the vaccine. Commercial chickens can be used in future study to truly reflect the vaccine's immunogenicity in farm setting. Next, protective efficacy and post-challenge study should be conducted to fully evaluate the vaccine's efficacy. Last but not least, duplicates or triplicates for HI test should be performed to ensure consistency and reliability of the results obtained.

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