UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF RECENTLY ISOLATED NEWCASTLE DISEASE VIRUSES AND DEVELOPMENT OF INACTIVATED VACCINE USING GENOTYPE VII NEWCASTLE DISEASE VIRUS

ODAY ABDUL RAZZAQ ABDUL WAHHAB ALJUMAILI

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By

ODAY ABDUL RAZZAQ ABDUL WAHHAB ALJUMAILI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

October 2017

DEDICATION

The sake of Allah, my Creator and my Master,

My great teacher and messenger, Mohammed (May Allah bless and grant him), who taught us the purpose of life.

Am dedicating this thesis to two beloved people who have meant and continue to mean so much to me. Although they are no longer of this world, their memories continue to regulate my life.

First and foremost, to my Father whose love for me knew no bounds and, who taught me the value of hard work. Thank you so much "Haj Abdulrazzaq", I will never forget you.

I also want to remember my lonely brother "Ammar" who loved me, and supported me whose life was cut short at the tender age of 43. He has gone forever away from our loving eyes and who left a void never to be filled in our lives. Though your life was short, I will make sure your memory lives on as long as I shall live. I love you all and miss you all beyond words. May Allah (Subhanaho Wa Tallah) grant all of you Jannahtul AlFirdaws.

Amen.

To my mother, who continues praying to me, supporting and developing and who has been a source of encouragement and inspiration to me throughout my life, very special thanks for her.

My dearest wife, who leads me through the valley of darkness
with light of hope and support,
My beloved sisters;
My beloved kids: Mostafa, Dhuha, and Ghena whom I can't force myself
to stop loving. To all my family, the symbol of love and giving,
My friends who encourage and support me,
All the people in my life who touch my heart,
I dedicate this research to you .

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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ODAY ABDUL RAZZAQ ABDUL WAHHAB ALJUMAILI

October 2017

Chairman : Professor Aini bt Ideris, PhD
Faculty : Veterinary Medicine

Newcastle Disease (ND) is a highly contagious and economically devastating disease of poultry in many parts of the world. At present, limited molecular epidemiological data are available regarding the causes of ND outbreaks in vaccinated chickens in commercial poultry farms. Knowing the genomic characteristics of Newcastle disease virus (NDV) infecting commercial poultry operations despite the chickens were vaccinated may give important insights on the infection dynamics of these viruses. In addition, molecular analyses at the subgenotype level and studies on the relationship of Malaysian NDVs with other isolates from around the world are lacking. Although many countries, including Malaysia maintain a stringent vaccination policy against ND, there are indications that ND outbreaks can still occur despite intensive vaccination. Virulent genotype VII NDV from China, Indonesia, Korea and Malaysia share only 82 to 87% similarity in amino acid residues of F and HN antigens respectively with B1 and LaSota vaccine strains (genotype II). While these genotype II-based vaccines prevent disease, they cannot stop viral shedding in the environment. Hence, the need for the so-called genotype-matched vaccines, which have been shown to reduce virus shedding, compared to genotype-mismatched vaccines is highly anticipated. Therefore, in the present study, a molecular epidemiological investigation is conducted to characterize six NDVs isolated from vaccinated commercial poultry flocks. To better understand the epidemiology of Newcastle disease outbreak, a partial F gene and HN gene were amplified from UPM-IBS 046/2014, UPM-IBS 060/2014, UPM-IBS 061/2014, UPM-IBS 074/2014, UPM-IBS 160/2015, and UPM-IBS 162A/2015 isolates by using conventional one step reverse

transcription-polymerase chain reaction (RT-PCR) and then conducted sequence and phylogenetic analysis. Furthermore, inactivation of NDV IBS/025/2013 strain (naturally recombinant strain) by two different method i.e. Ultraviolet type C (UVC) and Binary ethylinimine (BEI), and tested the inactivation by inoculation of the inactivated virus in SPF eggs for two passages was successfully executed.

Six NDV isolates which were recovered from ND outbreaks in chicken flocks in Malaysia were genotypically characterized. All the isolates had close phylogenetic relationship with previously characterized isolates from Malaysia as well as different countries within genotype VIIa, and genetically on the basis of the fusion (F) protein cleavage site. Among these, six NDV isolates showed an F protein cleavage site motif ^{112}RRQKRF^{117} ,^{112}KRRKRF^{117} ,^{112}KRRKRF^{117}, ^{112}KRRKRF^{117} ^{112}KRRKRF^{117},and ^{112}KRRKRF^{117}.

In the present study, NDV IBS/025/2013 strain (a naturally recombinant virus strain) was successfully inactivated by UVC light+Riboflavin and chemical (BEI), and then passaged consecutively two times in SPF chicken embryonated eggs. Results from the virus inactivation study revealed that exposure to UVC light for 14 hours or treatment of the virus with BEI for 21 hours at 37^{0}C successfully inactivated the virus as evidenced by its inability to kill SPF chicken embryonated eggs 6 days post-inoculation in two consecutive passages. Both inactivated vaccines were emulsified in two different adjuvants, *Nigella sativa* oil adjuvant, and Freund's incomplete adjuvant to produce vaccines in the form of water in oil (W/O). A stable W/O vaccine with two different adjuvants was successfully formulated. To test the efficacy of each vaccine formulation, ten days old SPF chickens were randomly divided into 11 groups, with 11 chickens in each group. The first 5 groups were vaccinated with different formulations of the vaccine, but the remaining groups were vaccinated with LaSota live vaccine (+L) in different formulations. One group was kept as control. The result for in vivo experiment indicated that the BEI-black seed oil (BEI-BSO), BEI- Incomplete Freund's adjuvant (BEI-IFA) as well as commercial vaccine, were fully protective against a virulent NDV challenge. Although all vaccinated groups had significantly lower mortality rate than unvaccinated-challenged group, full protection from death was observed in birds of group BEI-BSO, BEI-IFA, Commercial, UVC-BSO+L, UVC-IFA+L, BEI-BSO+L, BEI-IFA+L, and Commercial +L followed by group UVC-IFA with 33% mortality. Among vaccinated groups, the best results with regards to clinical signs and gross lesions were obtained in groups that were vaccinated with killed and live vaccine together, and groups of killed vaccine especially commercial and BEI-IFA; followed by group BEI-BSO with 10% morbidity. Vaccination provided high HI antibody titers in most of the vaccinated groups excluding UVC-BSO

and UVC-IFA. The chickens in all vaccinated-challenged groups shed the challenge virus from day 3 days post challenge. Insignificant differences were observed in the frequencies of virus detection among vaccinated groups (UVC-BSO and UVC-IFA) and positive control with different incidence. Duration of virulent virus shedding in infected birds of vaccinated groups were different. Vaccination programs used in groups of killed and live vaccine have shortened the duration of virus shedding (only at 3 days post challenge), while control group and UVC-BSO group started from day 3 post challenge continued to shed the virulent virus in the feces until the end of the experiment. Meanwhile the UVC-IFA started to shed the virus from day 5 post challenge until the end of the experiment. Finally, the BEI-BSO, BEI-IFA and commercial groups started to shed the virus from day 7 post challenge until the end of the experiment with reduced titer at 10days post challenge. The measurement of potency for the inactivated vaccine BEI-BSO and BEI-IFA by the use of the mortality as the metric, resulted in $10^{-7.612}$ of the full dose for BEI-BSO while $10^{-7.532}$ was for BEI-IFA.

In conclusion, the etiologic agents of the ND outbreaks recently reported in vaccinated chickens in Malaysia were found belonging to the velogenic genotype VIIa strain. The exposure of the NDV to UVC light + riboflavin for 14 hours or the exposure of the virus to BEI treatment for 21 hours at 37^{o}C was found to be adequate for the complete inactivation of the virus as demonstrated by its failure to induce mortality in the SPF chicken embryonated eggs, 6 days post inoculation in two successive passages. The preparation of stable water in oil emulsion from both black seed oil and incomplete Freund's adjuvant was successfully achieved in this study. An inactivated ND oil-emulsified vaccine from NDV IBS/025/13 high pathogenic viruses provides protection in young chickens against NDV IBS 002/11 genotype VII virus isolate. The BEI-black seed oil and BEI- Freund's adjuvant as well as commercial vaccine were demonstrated in this study to be capable of offering full protection against virulent NDV challenge.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENCIRIAN VIRUS PENYAKIT NEWCASTLE YANG DIPENCILKAN BARU-BARU INI DAN PEMBANGUNAN VAKSIN TIDAK AKTIF MENGGUNAKAN VIRUS PENYAKIT NEWCASTLE GENOTIP VII

Oleh

ODAY ABDUL RAZZAQ ABDUL WAHHAB ALJUMAILI

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Penyakit Newcastle (ND) ialah sejenis penyakit yang amat mudah berjangkit dan mengakibatkan kemusnahan dari segi ekonomi di kebanyakan bahagian di dunia ini. Pada masa kini, data epidemiologi molekular berkenaan penyebab wabak ND di ladang ternakan ayam komersil yang telah divaksinasi adalah terhad. Pengetahuan berkenaan ciri-ciri genomik virus penyakit Newcastle (NDV) yang menjangkiti operasi ternakan ayam komersil meskipun selepas vaksinasi mampu memberikan tanggapan yang penting berkenaan dinamik jangkitan virus ini. Tambahan pula, analisis molekular pada tahap subgenotip dan kajian berkenaan hubungan NDV di Malaysia dengan pencilan lain di seluruh dunia adalah terhad. Walaupun kebanyakan negara-negara, termasuk Malaysia mengekalkan polisi vaksinasi yang ketat terhadap ND, terdapat tanda-tanda bahawa wabak ND masih boleh berlaku sungguhpun vaksinasi intensif telah dijalankan. NDV genotip VII virulen dari Cina, Indonesia, Korea, dan Malaysia berkongsi hanya 82–87% persamaan dalam residu asid amino pada antigen-antigen F dan HN masing-masing dengan strain vaksin B1 dan LaSota (genotip II). Walaupun vaksin berasaskan genotip II ini mampu menghalang penyakit, ia tidak berupaya menghalang peluruhan virus ke persekitaran. Oleh yang demikian, keperluan bagi vaksin berpadanan-genotip, yang telah berjaya menunjukkan pengurangan peluruhan virus, berbanding vaksin tak berpadanan-genotip adalah sangat diharapkan. Oleh itu, di dalam kajian ini, satu penyelidikan epidemiologi molekular dijalankan bagi mencirikan enam NDV yang telah dipencilkan dari kumpulan ternakan ayam komersil yang divaksinasi. Bagi lebih memahami epidemiologi wabak penyakit Newcastle, gen separa F dan gen HN telah

diamplifikasi daripada pencilan-pencilan UPM-IBS 046/2014, UPM-IBS 060/2014, UPM-IBS 061/2014, UPM-IBS 074/2014, UPM-IBS 160/2015, dan UPM-IBS 162A/2015 dengan menggunakan kaedah lazim satu langkah tindakbalas rantaian polimerase-transkripsi berbalik (RT-PCR), dan kemudian pengendalian analisis jujukan dan filogenetik. Selanjutnya, penyahaktifan strain IBS 025 (strain rekombinan semulajadi) melalui dua kaedah berbeza iaitu UVC dan BEI, dan pengujian penyahaktifan dengan menginokulasi virus tidak aktif itu ke dalam telur SPF sebanyak dua laluan telah berjaya dilakukan.

Enam pencilan NDV yang telah didapati dari wabak ND pada kumpulan ayam di Malaysia telah dicirikan secara genotip. Kesemua pencilan tersebut mempunyai hubungan filogenetik yang rapat dengan pencilan yang telah dicirikan terdahulu di samping negara-negara berbeza dalam genotip VIIa, dan secara genetik pada asas tapak belahan protein lakuran (F). Di antara kesemuanya, enam pencilan-pencilan NDV menunjukkan motif tapak belahan protein F ^{112}RRQKRF^{117} ,^{112}KRRKRF^{117} ,^{112}KRRKRF^{117}, ^{112}KRRKRF^{117} ^{112}KRRKRF^{117}, dan ^{112}KRRKRF^{117} masing-masing.

Selanjutnya, dalam kajian ini, strain IBS 025/13 (strain rekombinan semulajadi) telah berjaya dinyahaktifkan dengan lampu UVC+Riboflavin dan bahan kimia (BEI), dan telah dilalukan berturutan sebanyak dua kali dalam telur ayam berembrio SPF. Keputusan dari kajian ketidakaktifan virus menunjukkan bahawa pendedahan kepada lampu UVC selama 14 jam atau rawatan virus dengan BEI selama 21 jam pada 37°C telah berjaya menyahaktifkan virus tersebut sebagaimana dibuktikan dengan ketidakupayaannya untuk mematikan telur ayam berembrio SPF pada 6 hari pos-inokulasi dalam dua laluan berturutan. Kedua-dua vaksin tidak aktif tersebut telah diemulsifikasikan dalam dua adjuvan berbeza, adjuvan minyak *Nigella sativa,* dan adjuvan tak lengkap Freund's bagi menghasilkan vaksin dalam bentuk air dalam minyak (W/O). Tambahan lagi, satu vaksin W/O yang stabil dengan dua adjuvan berbeza telah berjaya diformulasi. Bagi menguji keberkesanan setiap formulasi vaksin, ayam SPF berumur sepuluh hari telah dibahagikan secara rawak kepada 11 kumpulan, dengan 11 ekor ayam dalam setiap kumpulan. Lima kumpulan pertama telah divaksinasi dengan formulasi vaksin yang berbeza, tetapi kumpulan selanjutnya telah divaksinasi dengan vaksin hidup LaSota (+L) dalam formulasi berbeza. Satu kumpulan dikekalkan sebagai kawalan. Keputusan bagi eksperimen in vivo menunjukkan bahawa minyak BEI-bijan hitam (BEI-BSO), BEI-adjuvan tak lengkap Freund's (BEI-IFA) di samping vaksin komersil, adalah melindungi sepenuhnya daripada cabaran NDV virulen. Walaupun kesemua kumpulan divaksinasi mempunyai kadar kematian lebih rendah yang ketara berbanding kumpulan cabaran-tak divaksinasi, perlindungan penuh daripada kematian

telah diperhatikan pada ayam dalam kumpulan BEI-BSO, BEI-IFA, komersil, UVC-BSO+L, UVC-IFA+L, BEI-BSO+L, BEI-IFA+L, dan komersil +L diikuti dengan kumpulan UVC-IFA dengan 33% kematian. Di kalangan kumpulan divaksinasi, keputusan paling baik berkenaan tanda-tanda klinikal dan lesi kasar didapati dari kumpulan yang divaksinasi dengan vaksin terbunuh dan hidup bersama-sama, dan kumpulan vaksin terbunuh terutamanya komersil dan BEI-IFA; diikuti dengan kumpulan BEI-BSO dengan 10% morbiditi. Vaksinasi menyebabkan titer antibodi HI yang tinggi pada kebanyakan kumpulan divaksinasi melainkan UVC-BSO dan UVC-IFA. Ayam di dalam kesemua kumpulan cabaran-vaksinasi meluruhkan virus cabaran dari hari ketiga pos cabaran. Perbezaan yang tidak ketara diperhatikan dalam kekerapan pengesanan virus di kalangan kumpulan divaksinasi (UVC-BSO dan UVC-IFA), dan kawalan positif dengan kejadian berbeza. Tempoh peluruhan virus virulen pada ayam dijangkiti dalam kumpulan divaksinasi adalah berbeza. Program vaksinasi yang digunakan pada kumpulan vaksin terbunuh dan hidup telah memendekkan tempoh peluruhan virus (hanya pada hari ketiga pos cabaran), manakala kumpulan kawalan dan UVC-BSO bermula dari hari ketiga pos cabaran berterusan meluruhkan virus virulen di dalam najis hingga ke akhir eksperimen. Sementara itu, kumpulan UVC-IFA mula meluruhkan virus dari hari kelima pos cabaran hingga ke akhir eksperimen. Akhirnya, kumpulan-kumpulan BEI-BSO, BEI-IFA dan komersil mula meluruhkan virus dari hari ketujuh pos cabaran hingga ke akhir eksperimen dengan penurunan titer pada hari kesepuluh pos cabaran. Pengukuran potensi vaksin dinyahaktif BEI-BSO dan BEI-IFA dengan menggunakan kematian sebagai metrik, memberikan keputusan $10^{-7.612}$ untuk dos lengkap BEI-BSO manakala $10^{-7.532}$ untuk BEI-IFA.

Sebagai kesimpulan, agen etiologi wabak ND yang dilaporkan baru-baru ini pada ayam divaksinasi di Malaysia tergolong dalam strain velogenik genotip VIIa. Pendedahan NDV tersebut pada lampu UVC+riboflavin selama 14 jam atau perawatan virus tersebut dengan rawatan BEI selama 21 jam pada 37°C ditemui adalah mencukupi bagi penyahaktifan lengkap virus sebagaimana ditunjukkan dengan kegagalannya untuk menyebabkan kematian pada telur ayam berembrio SPF, 6 hari pos inokulasi dalam dua laluan berturutan. Penyediaan emulsi stabil air dalam minyak daripada kedua-dua minyak bijan hitam dan adjuvant Freund's tak lengkap telah berjaya dicapai dalam kajian ini. Satu vaksin ND tidak aktif minyak-teremulsi daripada virus sangat patogenik IBS/025 berupaya melindungi ayam muda terhadap pencilan virus IBS/002 genotip VII. Minyak BEI-bijan hitam dan BEI-adjuvan Freund's di samping vaksin komersil telah dibuktikan dalam kajian ini berupaya menawarkan perlindungan sepenuhnya terhadap cabaran NDV virulen.

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So thank you all.

I certify that a Thesis Examination Committee has met on 26 October 2017 to conduct the final examination of Oday Abdul Razzaq Abdul Wahhab Aljumaili on his thesis entitled "Characterization of Recently Isolated Newcastle Disease Viruses and Development of Inactivated Vaccine using Genotype VII Newcastle Disease Virus" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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Declaration by graduate student

I hereby confirm that:

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Declaration by Members of Supervisory Committee

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

aa	Amino Acid
APMV	Avian Paramyxovirus
BLAST	Basic Local Alignment Search Tool
BSO	Black seed oil
DNA	Deoxyribonucleic Acid
dpc	Day post challenge
dsRNA	Double strand Deoxyribonucleic Acid
EID_{50}	Embryo infective dose 50
ELISA	Enzyme-linked immunosorbent assays
F	Fusion Protein
F0	Fusion Protein 0
F1	Fusion Protein 1
F2	Fusion Protein 2
GC-MS	Gas chromatography–mass spectrometry
GIT	Gastrointestinal Tract
HI	Hemagglutination Inhibition
HN	Hemagglutinin-Neuraminidase
IBD	Infectious Bursal Disease
ICPI	Intra Cerebral Pathogenicity Index
IFA	Incomplete Freund's adjuvant
IVPI	Intra Venus Pathogenicity Index
L	Large Polymerase Protein
M	Matrix Protein
MEGA	Molecular Evolutionary Genetics Analysis
mM	Millimolar
NCBI	National Centre for Biotechnology Information
ND	Newcastle Disease
NDV	Newcastle Disease Virus
nm	Nano meter
NP	Nucleocapsid Protein
NV-ND	Neurotropic Velogenic Newcastle Disease
O/W	Oil in water
OIE	Office International des Epizooties
P	Phosphoprotein Protein
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD60	Protective dose 60
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
RT	Reverse Transcription
RT-PCR	Reverse Transcription-Polymerase Chain Reaction

SPF	Specific-Pathogen-Free
TAE	Tris-Acetate-EDTA
UVC	Ultraviolet light type C
VG/GA	Villegas-Glisson/University of Georgia
VV-ND	Viscerotropic Velogenic Newcastle Disease
W/O	Water in oil
W/O/W	Water in oil in water

CHAPTER 1

INTRODUCTION

1.1 Introduction

Since over 80 years of the Newcastle disease (ND) discovery in Java and England (Doyle, 1927; Kraneveld, 1926) and vaccine against NDV introduction in 1950s in controlling NDV disease (Alexander and Senne, 2008), Newcastle disease is yet the one of the most significant avian infections which lingers and causes high loss economically in the poultry industry. Due to its global epidemics and geographical spread, the Organization for Animal Health (OIE) of the world enlisted Newcastle disease to be a notifiable disease (OIE 2013). Newcastle disease is as a result of infection with Newcastle disease virus (NDV). This virus is an extremely infectious agent which is able to cause high dead rate in unvaccinated flocks, beside the subclinical forms of Newcastle disease in flocks that are vaccinated and/or Newcastle disease virus exposed flocks which might possess synergist consequence with other viral and or bacterial infections which can lead to additional severe disease and higher losses economically (Swayne and King, 2003).

Newcastle disease virus (NDV) is an avian paramyxovirus serotype-1 (APMV-1) which is a member of sub-family Paramyxovirinae from the family Paramyxoviridae and in the Mononegavirales virus order (Fauquet and Fargette, 2005; Mayo, 2002). NDV possess a negative sense, single stranded RNA genome that encodes for 6 genes (Lyles et al., 2013). Even though the entire APMV-1 viruses comes from a single serotype, they possess different genomic structures and are separated into different genotypes (Diel et al., 2012). Newcastle disease viruses are classified due to their F gene to 2 basic classes. Class one (I) Newcastle disease viruses are typically isolated from water fowls (Anatidae) and shore birds whereas, Class two (II) Newcastle disease viruses induces disease in poultry, and are alienated into ten (10) different genotypes (Miller et al., 2010). Certain infectious Newcastle disease virus which are isolates from several nations within Europe during 1990s were notice not to fit into whichever of recognized genotypes of those time, therefore, were categorized as genotype VII NDVs (Lomniczi et al., 1998). These Newcastle disease viruses are thought to be from East-Asia (Lomniczi et al., 1998), and subsequently extent to Middle-East, Europe and Africa (Bogoyavlenskiy et al., 2009; Wang et al., 2006). Recently research revealed that currently genotype VII is the main circulating NDV in South-East Asia which causes main epidemics comprising vaccinated flocks against Newcastle disease virus (Umali et al., 2013; Yi et al., 2011; Tan et al., 2010; Cho et al., 2008) .

From the time when NDV was discovered, countless determinations have been executed in controlling Newcastle disease in poultry industry. Apart from biosecurity and practice of good farm husbandry, the control of Newcastle disease is by vaccinating the flocks. Presently, numerous diverse vaccines are accessible in the market for Newcastle disease control both in large layer/ broiler chicken farms and also chickens of backyard village. Majority of the vaccines is from genotype II of class II of Newcastle disease viruses (Chong et al., 2010). Malaysia is like many other nations, poultry farmers utilized low pathogenic Newcastle disease viruses viz. lentogenic NDV such as Hitchner B1 and LaSota, as live vaccines for the protection against Newcastle disease. Apart from the two live vaccines, other Newcastle disease virus vaccine strains for example S, Ulster 2C, NDV-6/10 and enteric vaccine strain VG-GA is also been utilized (Aini, 2006). Numerous different types of genetically produced ND vaccines have been produced and tried experimentally. Nevertheless, very limited recombinant Newcastle disease virus vaccines are commercially obtainable, viz., herpesvirus turkey virus (HVT) based Newcastle disease virus vaccine (Palya et al., 2012). These vaccines have displayed encouraging outcomes in rendering defense against experimental trial with Newcastle disease virus velogenic strain.

Even though it is likely to evaluate vaccine efficiency by laboratory scale experimentations, it is very challenging to estimate the vaccine efficiency in the field (Chulan et al., 1982). Therefore, failure of vaccination after Newcastle disease virus vaccination has been stated. Amongst the primary issues that help to poor Newcastle disease virus vaccine induced immunity are incorrect vaccination dosage and timing, existence of simultaneous infection specifically immunosuppressive agents for example chicken infectious anemia (CAV), infectious bursal disease (IBD) or Marek's disease, nutritive insufficiencies in addition to mycotoxins in feed being the likely cause (s) for the breakdown in the vaccine inducing protection (Habibian et al., 2014; Zhang et al., 2012; Saif, 1991). Nonetheless, current research revealed that, commercially obtained Newcastle disease virus vaccines offer diverse level of protection against challenged with diverse genotypes of Newcastle disease virus (Hu et al., 2009; Miller et al., 2009) rising the status of affinity between vaccine and field strains of NDV. Additionally, it has been established that, LaSota vaccine, a genotype II NDV is not efficient in decreasing shedding of virus and clinical symptom after experimental trial with genotype VII isolates as equated to the reverse genetic designed genotype VII vaccine (Hu et al. 2009; Cho et al. 2008b). Nevertheless, the significance of virus shedding and transmission to vulnerable flocks is yet to be clear. Several research revealed that Newcastle disease virus vaccine was capable of protecting against death (disease immunity) nonetheless incapable in providing sterilizing immunity through prevention of infection after challenging with velogenic NDV (Cornax et al., 2012; Ezema et al., 2009).

Since the year 2000, genotype VII NDV has been reported in unvaccinated and vaccinated flocks of chicken in Malaysia (Berhanu et al., 2010; Tan et al., 2010). Recently investigation revealed that genotype VII NDV yet circulate amongst the chicken flocks in the country in spite of the widespread usage of LaSota based vaccines (Roohani et al., 2015). Nevertheless, inadequate investigation have being done in gaining access to the capability of genotype II (LaSota, B1, VG/GA, Avinew) (genotype mismatched vaccine) and genotype VII (genotype matched vaccine) in rendering defense against experimental trial with velogenic genotype VII in specific-pathogen-free (SPF) and large flocks of chicken.

The Malaysian strain (IBS025/13) has been characterized as natural recombinant strain between genotype II and genotype VII (Satharasinghe et al., 2016). It has nucleocapsid protein and phosphoprotein genes of genotype II whereas surface glycoproteins (fusion, hemagglutinin-neuraminidase) and large polymerase of genotype VII this strain has been used in this study to prepare the inactivated vaccines.

Typically, two leading types of vaccines are utilized in protecting animals and man clinically and virologically on experimental trials: improved live virus (attenuated virus) vaccines and killed virus (KV; inactivated virus) vaccines. KV vaccines are regularly chosen as a result of it safety reason, nevertheless certain problems could occur when using them. Initially, inactivation of virus could later reverse to be imperfect with epidemics post vaccination which could be a consequence (Patil et al., 2002; Beck and Strohmaier, 1987). Additionally, the viral-neutralizing epitopes could be damaged at the course of inactivation, resulting to reduced neutralizing antibody reaction and a reduced defense on experimental trial (Cham et al., 2006; FAO, 1995). Hence, effective excellence control of the inactivated antigen is essential in evaluating virus inactivation and the consequence of the inactivation process on neutralizing epitopes. However, currently existing viral vaccines are unable to cope with many types of prevailing viruses in the field. Therefore, new vaccines have to be created from the strains responsible for new outbreaks (Lee et al., 2012).

These days, commercial available killed vaccines are commonly primed from mineral oil as an adjuvant. The mineral oil can lead to opposing outcome plus tissue reaction; remains of vaccine in tissues of the poultry, the mineral could result to carcinogenic agent on the poultry and poultry products consumers. Due to the opposing properties of the mineral oil, considerable investigation had been introduced to overcome the difficulties through discovering other types of oil which could be replaced for the mineral oil (Stone, 1997; Gupta et al., 1993; Yamanaka et al., 1993). In the year 1996, inactivated vaccine in form

of water in oil in water (WOW) was developed through the use of subunit virus, acquired from whole or incomplete interruption by Tween 80, as an antigen (Cajavec et al., 1996). The vaccine delivered a less viscidness, high steadiness with lowered percentage of mineral oil in the vaccine to sustain the useful vaccination with stress-free washing of vaccination apparatus (Kaleta and Baldauf, 1988). Preceding report show that the WOW vaccine appeared to result in less reactions of tissue as compare to water in oil (WO) vaccine due to less liquid paraffin concentration (Fukanoki et al., 2001).

Vaccines produced from natural oil delivered lesser efficacy of stimulating HI antibody and higher viscosity as equated to vaccines produced from mineral oil (Stone, 1993).

1.2 Hypothesis

The hypothesis of this investigation is that

1- The genotype of Newcastle disease virus isolated from ND epidemics from vaccinated chicken farms belongs to velogenic genotype VII.
2- The inactivated NDV vaccine using naturally occurring recombinant genotype VII is able to induce better immune responses and protection against challenge with virulent genotype VII NDV.
3- The inactivated NDV vaccine formulated in black seed oil (*Nigella Sativa* oil) as adjuvant is able to induce better protection compared to inactivated NDV vaccine formulated in Freund's incomplete adjuvants against genotype VII NDV challenge.

To address the entire hypotheses, the specific objectives of this investigation are as follows:

1- To isolate and perform molecular characterization of Newcastle disease virus (NDV) from Newcastle disease outbreaks originated from NDV vaccinated farms.
2- To evaluate the inactivation of ND virus through different methods (UVC only, UVC+ Riboflavin and conventional BEI inactivation).
3- To study the adjuvant properties of black seed oil with incomplete Freund's adjuvant of inactivated ND vaccine in the form of W/O.
4- To determine the efficacy and potency of the developed NDV vaccines against velogenic genotype VII challenge in specific-pathogen-free flocks of chickens.

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