



UNIVERSITI PUTRA MALAYSIA

***CHARACTERIZATION OF NEWCASTLE DISEASE VIRUS (NDV)
ISOLATED FROM NDV VACCINATED BROILER FARMS AND
INVESTIGATION OF VACCINE EFFICACY AGAINST
CHALLENGE WITH VELOGENIC GENOTYPE VII NDV***

KIARASH ROOHANI SHAHRESTANI

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By

KIARASH ROOHANI SHAHRESTANI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra
Malaysia, in Fulfilment of the Requirements for the Degree of Master of
Science**

September 2014

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DEDICATION

This thesis is dedicated to my parents who have supported me all the way since the beginning of my life and my dear sister. Also, this thesis is dedicated to my beloved wife who has been a great source of motivation and inspiration.

Finally, this thesis is dedicated to all those who believe in the richness of learning.



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Master of Science

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September 2014

Chairman: Abdul Rahman Omar, DVM, PhD

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Vaccines to control Newcastle disease (ND) were introduced more than 60 years ago. Despite that, ND is still one of the most significant avian diseases affecting major poultry farms in various countries. Class II of Newcastle disease viruses (NDV) can be divided into 10 different genotypes based on the F gene. However, since 1990s genotype VII NDV is the predominant velogenic NDV circulating in South-East Asia causing outbreaks even in well vaccinated flocks. Various factors such as inappropriate vaccination scheme, concurrent infections, immunosuppression and presence of variant NDV have been implicated as the probable causes of the outbreak. The current NDV vaccines comprised of genotype I and/or II viruses. Through the use of reverse genetic technology, genotype-matched NDV vaccine has been used in certain countries. However, the efficacy of this vaccine against the circulating velogenic genotype VII NDV is not well characterized.

This study focused on the isolation and characterization of velogenic NDV from NDV vaccinated chickens in Malaysia. Sequencing and phylogenetic analysis based on the F gene of five NDV isolates (IBS001 to IBS005) showed that the viruses belong to genotype VII and sub-genotype VIId of NDV, with F cleavage site motif of $^{112}RRRKRF^{117}$. In addition, sequencing of the C-terminus of the HN gene revealed that, viruses lack extension and encoded a typical amino acid sequence length of virulent NDV. Hence, molecular characterization based on the F and HN genes indicated the viruses (IBS001 to IBS005) belong to velogenic genotype VII NDV.

One of the isolates, IBS002, was further characterized based on sequencing of the complete length of F and HN genes and pairwise comparisons between different genotypes. A maximum distance was detected between IBS002 and LaSota with nucleotide/amino acids variation between 17.71% to 18.67% for

F gene and 20.89% to 23.37% for HN gene. Nucleotide/amino acids variations of 8.79% to 9.77% for F gene and 9.17% to 11.60% for HN genes were detected between isolate IBS002 and genotype VII Dalguban N+ vaccine. In addition, IBS002 has a mean death time (MDT) of 51.2 hours and intracerebral pathogenicity index (ICPI) of 1.76, further confirming that the virus is a velogenic strain.

Both genotype matched (Dalguban N+) and mismatched (LaSota and Avinew) vaccines induced 100% protection against mortality and severe clinical symptoms following challenge with 10^5 ELD₅₀ of IBS002. Vaccinated chickens also showed significant ($P < 0.05$) lower pathogenicity scores although there was no significant ($P < 0.05$) difference among the vaccinated groups. However, sentinel birds of Avinew and Dalguban N+ groups showed lower pathogenicity score compared to sentinels in LaSota group ($P < 0.05$). Furthermore, Avinew and Dalguban N+ vaccinated chickens shed significantly ($P < 0.05$) less virus after challenge and the viral load decreased faster than LaSota group. Moreover, sentinel birds mortality in LaSota vaccinated and non-vaccinated groups were significantly ($P < 0.05$) higher than Avinew and Dalguban N+ vaccinated groups suggesting the importance of genotype matched vaccine (Dalguban N+) and enteric based NDV vaccine (Avinew) in inducing vaccine induced immunity. Vaccine that matched with the hemagglutination-inhibition (HI) test's antigen induced significantly ($P < 0.05$) higher antibody compared to vaccine from other genotypes where a 2 Log₂ difference were detected when genotype VII and genotype II NDV antigens were used to detect homologous and heterologous HI titers.

Immunophenotyping study showed significant increased ($P < 0.01$) in KUL-1+ macrophages in PBMCs and splenocytes of control challenged birds. On the other hand, CD3+/CD4+ and CD3+/CD8+ T cells in spleen of different vaccinated groups were increased upon challenge suggesting the possible involvement of these cells in curtailing virus replication.

In conclusion, isolated NDVs were classified as velogenic strains and belonged to genotype VIIId of class II of NDV. Both genotype matched and mismatched NDV vaccines were able to confer protection against challenge with velogenic genotype VII NDV. However, genotype matched and enteric based NDV vaccines seems to be able to confer a more complete protection against virus shedding and transmission to susceptible chickens following challenged with velogenic genotype VII NDV.

**Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Sarjana Sains**

**PENCIRIAN VIRUS PENYAKIT SAMPAR (NDV) DARI LADANG
AYAM PEDAGING YANG DISUNTIK VAKSIN NDV DAN SIASATAN
EFIKASI VAKSIN TERHADAP CABARAN DENGAN NDV GENOTIP
VII VELOGENIK**

Oleh

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Walaupun lebih 60 tahun, vaksin bagi mengawal penyakit sampar (ND) telah diperkenalkan, ND masih merupakan salah satu penyakit unggas yang mempunyai impak besar terhadap ladang ayam di setiap pelusuk negara. Virus penyakit sampar (NDV) kelas II boleh dibahagikan kepada 10 genotip yang berbeza berdasarkan kepada gen F. Walau bagaimanapun, sejak tahun 1990an NDV genotip VII adalah virus velogenik utama yang tersebar di Asia Tenggara dan menyebabkan wabak mahupun dalam ayam yang disuntik vaksin. Pelbagai faktor yang menyumbang kepada penularan wabak ini termasuklah skim vaksinasi yang tidak sesuai, jangkitan secara serentak, imunotindas dan juga kemunculan virus penyakit sampar (NDV) varian. Vaksin NDV yang ada pada masa kini terdiri daripada virus genotip I dan/atau genotip II. Walau bagaimanapun, melalui penggunaan teknologi genetik terbalik, vaksin NDV genotip-padanan telah digunakan di negara-negara tertentu. Tetapi, efikasi vaksin ini terhadap NDV genotip VII beredar velogenik tidak dicirikan dengan begitu jelas lagi.

Kajian ini bertumpu kepada pengasingan dan pencirian NDV velogenik daripada ayam yang disuntik vaksin di Malaysia. Penjujukan dan analisis filogenetik berdasarkan gen F daripada lima isolat NDV (IBS001 hingga IBS005) menunjukkan bahawa virus-virus ini terdiri daripada NDV genotip VII dan sub-genotip VIId dengan tapak belahan F bermotifkan ¹¹²RRRKRF¹¹⁷. Selain daripada itu, jujukan terminus C gen HN menunjukkan bahawa virus-virus ini tidak mempunyai lanjutan jujukan dengan panjang jujukan asid amino yang kerap ditemui bagi NDV virulen. Oleh itu, pencirian molekul berdasarkan gen F dan HN menunjukkan virus-virus ini (IBS001 hingga IBS005) tergolong dalam genotip VII NDV velogenik. Salah satu virus yang diasingkan, IBS002, telah dilanjutkan penciriannya berdasarkan jujukan lengkap gen F dan HN, dan seterusnya dibandingkan di antara genotip yang berbeza. Satu perbezaan jarak maksimum dikesan di antara IBS002 dan

LaSota dengan nukleotida/asid amino, daripada 17.71% kepada 18.67% bagi gen F dan 20.89% kepada 23.37% bagi gen HN. Variasi nukleotida/asid amino daripada 8.79% kepada 9.77% untuk gen F dan 9.17% kepada 11.60% bagi gen HN telah ditemui di antara IBS002 dan vaksin genotip VII Dalguban N+. Di samping itu, IBS002 mempunyai purata masa kematian (MDT) iaitu 51.2 jam dan indeks patogenisiti intraserebrum (ICPI) iaitu 1.76 seterusnya mengesahkan bahawa virus ini tergolong dalam kumpulan NDV velogenik.

Kedua-dua vaksin genotip-padanan (Dalguban N+) dan tidak padanan (LaSota dan Avinew), memberi perlindungan 100% terhadap kematian dan gejala klinikal yang teruk ekoran cabaran dengan IBS002 pada sukatan virus 10^5 ELD₅₀. Ayam yang disuntik vaksin menunjukkan bacaan skor patogenisiti rendah yang signifikan ($P < 0.05$), manakala tiada sebarang perbezaan yang signifikan ($P < 0.05$) didapati di kalangan kumpulan ayam yang disuntik vaksin. Walau bagaimanapun, ayam sentinel daripada kumpulan Avinew dan Dalguban N+ menunjukkan skor patogenisiti yang lebih rendah berbanding dengan sentinel dalam kumpulan LaSota ($P < 0.05$). Selain itu, ayam yang telah disuntik vaksin Avinew dan Dalguban N+ menunjukkan peluruhan virus yang rendah ($P < 0.05$) selepas cabaran dan kuantiti virus menurun dengan lebih cepat daripada kumpulan LaSota. Tambahan lagi, skor kematian ayam sentinel dalam kumpulan vaksin LaSota dan kumpulan yang tidak disuntik vaksin ($P < 0.05$) adalah sangat tinggi jika dibandingkan dengan Avinew dan Dalguban N+ mencadangkan kepentingan vaksin genotip-padanan (Dalguban N+) dan vaksin berasas enterik dalam mengaruh keimunan vaksin. Vaksin yang sepadan dengan antigen ujian perencatan-hemagglutinin (HI) menunjukkan titer antibodi yang lebih tinggi ($P < 0.05$) berbanding dengan vaksin dari genotip lain di mana perbezaan 2 Log_2 dikesan pada titer antibodi HI homolog dan heterolog bila virus genotip VII dan II digunakan sebagai antigen.

Kajian imunofenotip menunjukkan ayam disuntik vaksin LaSota mempunyai peratus makrofaj KUL-1+ dan IgM+ B limfosit yang lebih tinggi berbanding dengan kumpulan disuntik vaksin lain. Walaupun begitu, tiada perbezaan yang ketara dikesan pada kedua-dua sel T limpa, CD3+/CD4+ dan CD3+/CD8+ dikalangan kumpulan yang disuntik vaksin yang berbeza. Peningkatan yang ketara ($P < 0.01$) makrofaj KUL-1+ dalam PBMCs dan limpa telah dikesan dalam kumpulan kawalan yang dicabar. Sungguhpun begitu, peningkatan yang ketara pada jumlah sel T ($P < 0.01$) limpa dari ayam yang disuntik vaksin dan dicabar dengan IBS002 mungkin menunjukkan peranan penting sel ini dalam mengekang replikasi virus. Kesimpulannya, kedua-dua vaksin genotip-padanan dan tidak padanan dapat memberikan perlindungan terhadap cabaran dengan NDV genotip VII velogenik. Walau bagaimanapun, vaksin NDV padanan dan berasaskan enterik dapat memberikan perlindungan yang lebih lengkap terhadap peluruhan virus dan transmisi virus kepada perumah rentan.

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I certify that a Thesis Examination Committee has met on 23 September 2014 to conduct the final examination of Kiarash Roohani Shahrestani on his thesis entitled "Characterization of Newcastle Disease Virus (NDV) Isolated from NDV-Vaccinated Broiler Farms and Investigation of Vaccine Efficacy Against Challenge with Velogenic Genotype VII NDV" 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 Master of Science.

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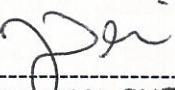
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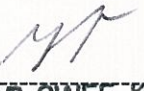
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LIST OF ABBREVIATIONS

Aa	Amino Acid
APMV	Avian <i>Paramyxovirus</i>
BLAST	Basic Local alignment Search Tool
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic Acid
dsRNA	Double strand Deoxyribonucleic Acid
EDTA	Ethylene-Diamine-Tetraacetic Acid
ELD ₅₀	Mean Egg Lethal Dose
ELISA	Enzyme-linked immunosorbent assays
F	Fusion Protein
F0	Fusion Protein 0
F1	Fusion Protein 1
F2	Fusion Protein 2
GIT	Gastrointestinal Tract
HI	Hemagglutination Inhibition
HN	Hemagglutinin-Neuraminidase
IBD	Infectious Bursal Disease
ICPI	Intra Cerebral Pathogenicity Index
IVPI	Intra Venus Pathogenicity Index
L	Large Polymerase Protein
M	Matrix Protein
MAB	Monoclonal Antibodies
MDT	Mean Death Time
MEGA	Molecular Evolutionary Genetics Analysis
NCBI	National Center for Biotechnology Information
ND	Newcastle Disease
NDV	Newcastle Disease Virus
NP	Nucleocapsid Protein
NV-ND	Neurotropic Velogenic Newcastle Disease
OIE	Office International des Epizooties
P	Phosphoprotein Protein
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
rHVT	Turkey Herpesvirus-based Recombinant Vaccine
RNA	Ribonucleic Acid
Rpm	Revolutions Per Minute
RT	Reverse Transcription
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SPF	Specific-Pathogen-Free

TAE
VG/GA
VV-ND

Tris-Acetate-EDTA
Villegas-Glisson/University of Georgia
Viscerotropic Velogenic Newcastle Disease



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CHAPTER 1

INTRODUCTION

After more than 80 years since the first discovery of Newcastle disease (ND) in England and Java (Kranefeld, 1926; Doyle, 1927) and the introduction of vaccine in 1950s to control the disease (Alexander, 2008), ND is still the most significant avian disease that continue to cause huge economical loss to the poultry industry. Because of its worldwide outbreaks and geographical spread, World Organization for Animal Health (OIE) has listed ND as a notifiable disease (OIE, 2013). The disease is caused by Newcastle disease virus (NDV), a highly infectious agent capable of causing high mortality in non-vaccinated chickens, beside the subclinical forms of ND in vaccinated and/or NDV exposure flocks which could have synergist effect with other bacterial or viral infections and cause more severe disease and bigger economic losses (Capua and Alexander, 2009).

Newcastle disease virus (NDV) is an avian paramyxovirus serotype-1 (APMV-1), a member of sub-family Paramyxovirinae of family Paramyxoviridae from virus order Mononegavirales (Mayo 2002; Fauquet and Fargette 2005). The virus has a negative sense, single stranded RNA genome which encodes for 6 genes (Lamb and Parks, 2006). Although all APMV-1 viruses are from one serotype, they share different genome structures and have been divided into different genotypes (Diel *et al.*, 2012). NDVs are categorized based on their F gene into 2 classes. Class I NDV were mostly isolated from water fowls (Anatidae) and shore birds whilst, Class II NDVs are able to induce disease in poultry, and are divided into 10 genotypes (Miller *et al.*, 2010). Some of virulent NDV isolates from different countries in Europe during 1990s were found not belonged into any of known genotypes by that days, hence, were classified as genotype VII NDVs (Lomniczi *et al.*, 1998). These NDVs are believed to be originated from East-Asia (Lomniczi *et al.*, 1998), later spread to Europe, Africa, Middle-East and South Africa (Wang *et al.*, 2006; Bogoyavlenskiy *et al.*, 2009). Recent studies have shown that presently genotype VII is the predominant circulating NDV in South-East Asia causing major outbreaks including in NDV vaccinated flocks (Cho *et al.*, 2008a; Tan *et al.*, 2010a; Yi *et al.*, 2011; Umali *et al.*, 2013).

Since the discovery of NDV, many efforts have been implemented to control ND in poultry industry. Beside biosecurity and good farm husbandry practices, ND is controlled through vaccination. Currently, several different vaccines are available commercially to control ND in both commercial chicken farms and backyard village chickens. Most of these vaccines belong to genotype II of class II of NDVs (Chong *et al.*, 2010). In Malaysia, like most of other countries, farmers are utilizing low pathogenic NDVs namely lentogenic NDV such as Hitchner B1 and LaSota, as live vaccines for ND prevention. Beside these two live vaccines, other NDV vaccine strains such

are S, Ulster 2C, NDV-6/10 and enteric vaccine strain VG-GA have also been used (Aini, 2006). Various type of genetically engineered ND vaccines have been developed and tested experimentally. However, only a few recombinant NDV vaccines are available commercially, namely, herpesvirus turkey virus (HVT) based NDV vaccine (Palya *et al.*, 2012). These vaccines have shown promising results in conferring protection against challenge with velogenic NDV.

Although it is possible to estimate vaccine efficacy through laboratory scale experiments, it would be very difficult to evaluate the vaccine efficacy in the field (Chulan *et al.*, 1982). Hence, vaccination failure following NDV vaccination has been reported. Among the underlying factors that contribute to poor NDV vaccine induced immunity are inappropriate vaccination dose and regime, presence of concurrent infection especially immunosuppressive agents such as chicken infectious anaemia (CAV), infectious bursal disease (IBD) or Marek's disease, nutritional deficiencies as well as mycotoxins in feed being the probably reason(s) for the break in the vaccine induced immunity (Saif, 1991; Zhang *et al.*, 2012; Habibian *et al.*, 2013). However, recent studies have shown that, commercial NDV vaccines provide different level of protections against challenged with different genotypes of NDV (Hu *et al.*, 2009; Miller *et al.*, 2009) raising the importance of relatedness between vaccine and field strains of NDV. Furthermore, it has been demonstrated that, LaSota vaccine, a genotype II NDV is not effective in reducing virus shedding and clinical signs upon challenge with genotype VII isolates compared to reverse genetic designed genotype VII vaccine (Cho *et al.*, 2008b; Hu *et al.*, 2009). However, the importance of virus shedding and transmission to susceptible chickens is not well defined. Numerous studies have shown that NDV vaccine was able to provide protection against mortality (disease immunity) but unable to provide sterilising immunity by preventing infection following challenged with velogenic NDV (Ezema *et al.*, 2009; Cornax *et al.*, 2012).

In Malaysia, since 2000, genotype VII NDV has been reported from both non-vaccinated and vaccinated chicken flocks (Maizan *et al.*, 2001; Berhanu *et al.*, 2010; Tan *et al.*, 2010a). Recent survey indicated that genotype VII NDV is still circulating among the poultry flocks in Malaysia despite the extensive use of LaSota based vaccines (unpublished data). However, limited studies have been carried out in accessing the ability of genotype II (LaSota, B1, VG/GA, Avinew) (genotype mismatched vaccine) and genotype VII (genotype matched vaccine) in conferring protection against challenge with velogenic genotype VII in specific-pathogen-free (SPF) and commercial chicken flocks.

Hence, the first hypothesis of this study is that the genotype of NDV isolated from ND outbreaks from vaccinated broiler farms belongs to velogenic genotype VII that is highly pathogenic in chickens. The second hypothesis of

this study is vaccination with genotype matched NDV will provide better protection against genotype VII NDV challenge.

To address both hypotheses, the specific objectives of this study are:

- To isolate and characterize Newcastle disease virus (NDV) from Newcastle disease outbreaks originated from NDV vaccinated farms based on biological and molecular analysis.
- To investigate the efficacy of genotype matched and genotype mismatched NDV vaccines against velogenic genotype VII challenge in specific-pathogen-free chickens based on serological, biological and immunological analysis.



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