UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF DNA VACCINE CONTAINING HN AND F GENES OF NEWCASTLE DISEASE VIRUS AND ITS EFFICACY FOLLOWING IN VIVO AND IN OVO IMMUNIZATIONS

MASOUMEH FIROUZAMANDI

FPV 2013 10

DEVELOPMENT OF DNA VACCINE CONTAINING HN AND F GENES OF NEWCASTLE DISEASE VIRUS AND ITS EFFICACY FOLLOWING IN VIVO AND *IN OVO* IMMUNIZATIONS

By

MASOUMEH FIROUZAMANDI

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

July 2013

COPYRIGHT

I would like to dedicate this thesis to my dearest parents, husband and my lovely son "Benyamin"

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

DEVELOPMENT OF DNA VACCINE CONTAINING HN AND F GENES OF NEWCASTLE DISEASE VIRUS AND ITS EFFICACYFOLLOWING IN VIVO AND *IN OVO* IMMUNIZATIONS

By

MASOUMEH FIROUZAMANDI

July 2013

Chairman: Professor Aini Bt Ideris, PhD

Faculty: Veterinary Medicine

Newcastle disease is a fatal viral disease which is highly contagious that affects most species of birds and is a major economic threat in the poultry industry. Both the HN and F glycoproteins of Newcastle disease virus (NDV) are essential for pathogenicity and virus infectivity. Though vaccines are available for the control of the disease but the vaccine with more efficacies is still required. The third generation vaccines, which include DNA vaccine, have the potential to be safer and more efficacious.

In this study, DNA vaccines developed successfully by using the HN and F genes from a Malaysian viscerotropic velogenic AF2240 NDV strain into the pIRES bicistronic mammalian expression vector separately and combined.

Three recombinant plasmids were constructed with the Kozak translation initiation sequences, namely, pIRES/HN, pIRES/F and pIRES/HN/F.

The HN and F genes in the pIRES vector has been expressed and tested successfully using the indirect immunofluorescence (IIF) test and Western blotting in the Vero cell line (in vitro). The results of IIF showed that all the DNA-transfected cells exhibited bright cytoplasmic fluorescence, indicating both the F and HN proteins were successfully expressed in the mammalian cell line. Also, Western blotting results revealed the expected bands of size approximately 74kDa, 55 kDa and 12 kDa for HN, F1 and F2, respectively.

In vivo experiment I showed that single vaccination with the plasmid DNA (pDNA) was not sufficient to induce antibody titer in specific pathogen free (SPF) chickens. Whereas, single pDNA vaccination and boosted by killed vaccine, showed a significant difference in ELISA antibody levels ($p<0.05$) elicited by either monocistronic (pIRES/HN + pIRES/F) or bicistronic (pIRES/HN/F) plasmids at one week post booster, compared with the killed vaccine alone. However, the HI titer was not significant higher ($p>0.05$) in vaccinated chickens with pIRES/HN/F and pIRES/HN+pIRES/F which boosted with killed vaccine at one week post booster compared with vaccinated chicken with killed vaccine alone. Overall, it was concluded that the recombinant pDNA vaccine can be used to increase the efficacy of the killed vaccine immunization procedure.

In vivo experiment II showed that twice vaccination with pDNA was able to elicit significant antibody titers ($p< 0.05$) by either monocistronic or bicistronic

plasmid, after one week of second pDNA vaccination (booster). The results proposed that DNA immunization of chickens at second vaccination had enhanced the antibody response successfully. As a result, the findings of the present study well demonstrated that vaccination with the co-expression plasmid pIRES/HN/F can induce a stronger antibody response than vaccination with pIRES/HN or pIRES/F alone.

In ovo vaccination with 10 µg pDNA/egg was not sufficient to induce production of antibody in SPF chickens. However, *in ovo* vaccination with 40 µg pDNA/egg induced high levels of antibody titer ($p<0.05$) in SPF chickens at four weeks post vaccination. The findings also showed that vaccination with 40 µg pDNA/egg able to confer protection against ND in two out of seven SPF chickens. Although, the chickens produced antibody titres three weeks post *in ovo* vaccination, it was not sufficient to protect the chickens from lethal viral challenge. However, vaccination with pDNA/Dextran-spermine complex did not induce high antibody titer compared with naked pDNA. In conclusion, DNA vaccination with pIRES/HN/F is suitable for in vivo application but not encourage for *in ovo* use.

More studies are required to investigate how to increase the efficacy of the constructed recombinant pDNA vaccines.

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

PEMBANGUNAN VAKSIN DNA MENGANDUNGI GEN HN DAN F VIRUS PENYAKIT NEWCASTLE DAN KEBERKESANANNYA SELEPAS IMUNISASI IN VIVO DAN IN OVO

Oleh

MASOUMEH FIROUZAMANDI

Julai 2013

Pengerusi: Profesor Aini Bt Ideris, PhD

Fakulti: Perubatan Veterinar

Penyakit Newcastle adalah sejenis penyakit disebabkan virus yang boleh membawa maut di mana ianya sangat mudah menular yang menjejaskan kebanyakan spesis burung dan adalah satu ancaman utama dalam industri penternakan ayam. Kedua-dua glikoprotein HN dan F pada virus penyakit Newcastle adalah penting untuk kepatogenan dan kejangkitan virus. Walaupun terdapat vaksin sedia ada untuk mengawal penyakit tersebut, vaksin yang lebih berkesan masih diperlukan. Vaksin-vaksin generasi ketiga, termasuk vaksin DNA, mempunyai keupayaan untuk menjadi lebih selamat dan berkesan.

Di dalam kajian ini, vaksin-vaksin DNA telah dibangunkan dengan jayanya menggunakan gen HN dan F daripada strain NDV viserotropik velogenik AF2240 dari Malaysia ke dalam bahagian pIRES bisistronik vektor pengekspresan mamalia secara berasingan dan bergabung. Tiga plasmid rekombinan telah dibuat dengan translasi jujukan permulaan Kozak, seperti, pIRES/HN, pIRES/F dan pIRES/HN/F.

Gen-gen HN dan F di dalam vektor pIRES telah berjaya dizahirkan dan diuji menggunakan ujian imunopendarfluor tidak langsung (IIF) dan pemendapan Western dalam jujukan sel Vero (in vitro). Keputusan daripada IIF menunjukkan bahawa semua sel-sel ditransfeksi-DNA mempamerkan pendarfluor sitoplasma yang bercahaya, menunjukkan bahawa kedua-dua protein F dan HN telah berjaya dizahirkan dalam jujukan sel mamalia tersebut. Malah, keputusan pemendapan Western menggambarkan jalur yang dijangkakan bersaiz lebih kurang 74kDa, 55 kDa dan 12 kDa untuk HN, F1 dan F2 masing-masing.

Eksperimen I in vivo, menunjukkan pemvaksinan satu kali dengan plasmid DNA (pDNA) tidak mencukupi untuk memulakan titer antibodi dalam ayam bebas penyakit khusus (SPF). Walau bagaimana pun, pemvaksinan satu kali dengan vaksin pDNA dan digalakkan dengan menggunakan vaksin terbunuh, menunjukkan perbezaan yang ketara dalam tahap antibodi ELISA ($P<0.05$), yang dibangkitkan sama ada oleh plasmid-plasmid monosistronik (pIRES/HN + pIRES/F) atau bisistronik (pIRES/HN/F) pada satu minggu selepas penggalakkan, berbanding dengan vaksin terbunuh sahaja. Walau

bagaimana pun, titer HI tidak tinggi secara ketara ($p>0.05$) dalam ayam-ayam yang telah divaksinasi dengan pIRES/HN/F dan pIRES/HN+pIRES/F yang telah digalakkan dengan vaksin terbunuh pada minggu pertama selepas penggalakan berbanding dengan ayam yang divaksinasi dengan vaksin terbunuh sahaja. Oleh itu, boleh diputuskan bahawa vaksin pDNA boleh digunakan untuk meningkatkan keupayaan prosedur imunisasi vaksin terbunuh tersebut.

Eksperimen II in vivo, menunjukkan vaksinasi dua kali dengan pDNA berupaya membangkitkan titer antibodi yang ketara ($P< 0.05$) sama ada oleh plasmid monosistronik atau bisistronik, selepas satu minggu pemvaksinan kedua pDNA kedua (penggalak). Keputusan-keputusan mencadangkan bahawa imunisasi DNA terhadap ayam-ayam pada vaksinasi kedua telah meningkatkan tindak balas antibodi dengan jayanya. Sebagai kesimpulannnya, keputusan-keputusan kajian ini dengan jelas menunjukkan bahawa vaksinasi dengan plasmid ekspresi-bersama pIRES/HN/F boleh mencetuskan tindak balas antibodi yang lebih kuat berbanding vaksinasi dengan pIRES/HN atau pIRES/F sahaja.

Vaksinasi *in ovo* dengan 10 µg pDNA/telur tidak mencukupi untuk mencetuskan antibodi dalam ayam-ayam SPF. Walau bagaimana pun, vaksinasi *in ovo* dengan 40 µg pDNA/telur mencetuskan tahap titer antibodi yang tinggi ($P<0.05$) pada minggu keempat selepas vaksinasi. Hasil penemuan juga menunjukkan bahawa vaksinasi dengan 40 µg pDNA/telur berupaya untuk mewujudkan perlindungan terhadap ND dalam dua daripada

tujuh ayam-ayam SPF. Walaupun ayam-ayam tersebut menghasilkan titer antibodi selepas tiga minggu vaksinasi in ovo, ianya masih tidak mencukupi untuk melindungi ayam-ayam tersebut daripada cabaran virus maut. Walau bagaimana pun, vaksinasi dengan pDNA/Dekstran-spermin tidak berupaya untuk mencetuskan titer antibodi yang tinggi berbanding pDNA terdedah. Sebagai kesimpulannya, vaksinasi DNA dengan pIRES/HN/F adalah sesuai digunakan dalam aplikasi *in vivo* tetapi tidak digalakkan bagi penggunaan *in ovo.*

Lebih banyak kajian diperlukan bagi menyelidik bagaimana untuk meningkatkan keberkesanan vaksin rekombinan pDNA yang telah dibina.

ACKNOWLEDGEMENTS

In the name of Allah, the Most Benevolent and Most Merciful

I would like to express my sincere gratitude to my major supervisor Prof. Datin Paduka Dr. Aini Ideris, for her invaluable guidance and unflinching support throughout this project. I thank my committee members, Professor Dr Mohd Hair Bejo, Professor Dr Abdul Rahman Omar, and Dr Seyed Davood Hosseini for their valuable input, time and support during my study.

My sincere appreciation goes to Dr. Parvaneh Merhbod and Dr. Hasan Moeini for their friendship, encouragement and timely help during various phases of my work. I would also like to thank the faculty and staff of the Faculty of Veterinary Medicine, Institute of Bioscience, and Razi Vaccine and Serum Research Institute for their cooperation and support during my period of study.

Special thanks goes to Dr. Tan Sheau Wei, Puan Rodiah Husin, Dr. Yeap Swee Keong and Mis. Haryati Shila for their kind help.

Finally, I am indebted to dear my parents, my husband Esmaeil, and all my family member for their love, understanding and moral support.

Approval

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Aini Ideris, PhD
Professor
Faculty Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Mohd Hair B Bejo, PhD
Professor
Faculty Veterinary Medicine
Universiti Putra Malaysia
(Member)

Abdul Rahman Omar, PhD
Professor
Faculty Veterinary Medicine
Universiti Putra Malaysia
(Member)

Seyed Davood Hosseini, PhD
Assistant Professor
Razi Vaccine and Serum Research Institute, Arak, Iran
(Member)

_____

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

MASOUMEH FIROUZAMANDI

Date:

TABLE OF CONTENTS

		Page
ABSTRACT		iv
ABSTRAK		vii
ACKNOWLEDGEMENTS		xi
APPROVAL		xii
DECLARATION		xiv
LIST OF TABLES		xix
LIST OF FIGURES		xx
ABBREVIATIONS		xxiii
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	6
	2.1 Newcastle Disease	6
	2.2 Newcastle Disease Virus (NDV)	8
	2.3 Immune Response to NDV	18
	2.4 Approach to Development of DNA Vaccines	22
	2.5 Approach to Development of DNA Vaccines Against NDV	25
	2.6 *In Ovo* Vaccination Against NDV	29
	2.7 Dextran-Spermine Nanoparticles as Novel Carrier Systems	34
3	**CONSTRUCTION OF BIVALENT DNA PLASMID CONTAINING THE HN AND F GENES OF MALAYSIAN VELOGENIC NDV**	38
	3.1 Introduction	38
	3.2 Materials and Methods	39
	3.2.1 Preparation of Velogenic Newcastle Disease Virus AF2240	39
	3.2.2 Viral RNA Extraction	40
	3.2.3 Concentration Determination of the Extracted RNA	41
	3.2.4 Primer Design	41
	3.2.5 Reverse Transcription and Polymerase Chain Reaction	42
	3.2.6 Detection and Purification of PCR Products	44
	3.2.7 Overview of pJET1.2/blunt Cloning Vector	44
	3.2.8 Overview of pIRES	45
	3.2.9 Stock Preparation of pIRES	46
	3.2.10 TA Cloning	48
	3.2.11 Confirmation of Gene Insertion by PCR	49
	3.2.12 Enzyme Digestion of the Recombinant Plasmids	50

3.2.13 Sub-Cloning of the HN and F Genes into pIRES plasmid p 50
3.2.14 Sequencing Analysis of the Recombinant pIRES plasmids 51
3.2.15 Stock Culture Preparation Recombinant Plasmids 52
3.3 Results and Discussion 52
3.3.1 RT-PCR Amplification of HN and F Genes 52
3.3.2 Purification of RT-PCR Products 55
3.3.3 Verification of the Recombinant Plasmids pJET/HN and pJET/F 56
3.3.4 Verification of Recombinant PlasmispIRES/HN and pIRES/F 58
3.3.5 Sequence Analysis of the pIRES/HN Plasmid 64
3.3.6 Sequence Analysis of the pIRES/F Plasmid 65
3.4 Conclusion 68

4 IN VITRO EXPRESSION OF HN AND F GENES OF VELOGENIC NDV (pIRES/HN, pIRES/F and pIRES/HN/F) IN VERO CELLS 69
4.1 Introduction 69
4.2 Materials and Methods 70
4.2.1 Cell Culture Methods 70
4.2.2 Cultivation of Vero Cell from Frozen Stocks 71
4.2.3 Maintenance of Vero Cell Culture 72
4.2.4 Preparation Frozen Stocks from Vero Cell 72
4.2.5 Transfection of Adherent Vero Cells by Cationic Lipid 73
4.2.6 RNA Extraction and RT-PCR 75
4.2.7 Indirect Immunofluoresence Test (IIFT) 76
4.2.8 Sample Preparation for SDS-PAGE and Western Blotting 77
4.2.9 SDS Polyacrylamide Gel Electrophores 78
4.2.10 Immunoblotting (Western Blotting) 79
4.3 Results and Discussion 80
4.3.1 Transcriptional Expression Analysis In Vitro 80
4.3.2 Indirect Immunofluorescence Test (IIFT) 81
4.3.3 Western Blotting (Immunoblotting) 82
4.4 Conclusion 85

5 IMMUNOGENICITY OF CONSTRUCTED PLASMIDS (pIRES/HN, pIRES/F and pIRES/HN/F) VACCINES IN SPF CHICKENS 86
5.1 Introduction 86
5.2 Materials and Methods 88
5.2.1 Preparation of the DNA Plasmids for In 88

Vivo Vaccination
5.2.2 DNA Immunization Program 88
5.2.3 Haemagglutination (HA) Assay 91
5.2.4 Haemagglutination Inhibition (HI) Assay 93
5.2.5 Detection of Anti-NDV Specific Antibodies 94
5.2.6 Statistical Analysis 94
5.3 Results and Discussion 95
5.3.1 NDV-HN Antibody Response In Experiment I 95
5.3.2 NDV Antibody Response In Experiment I 96
5.3.3 NDV-HN Antibody Response In Experiment II 98
5.3.4 NDV Antibody Response In Experiment II 99
5.4 Conclusion 102

6 OPTIMIZING A NANOPARTICLE DELIVERY SYSTEM OF DNA VACCINE (pIRES/HN, pIRES/F and pIRES/HN/F) FOR *IN OVO* VACCINATION 103
6.1 Introduction 103
6.2 Materials and Methods 106
6.3.1 Immunization of SPF Embryonated Eggs by Naked pDNA 106
6.2.1 Evaluation Evaluation of Humoral Immune Response by ELISA 106
6.2.2 Haemagglutination Inhibition (HI) Assay 107
6.2.3 Preparation of pDNA/Dextran-spermine Complexes 107
6.2.4 *In ovo* Vaccination of SPF Embryonated Eggsby Delivery pDNA/Dextran-spermine 109
6.2.5 ELISA and HI in SPF Embryonated Vaccinated with pDNA/Dxtran-spermine 111
6.2.6 Virus Challenge Experiments 111
6.2.7 Statistical Analysis 111
6.3 Results and Discussion 112
6.3.1 ELISA Antibody titres after Immunization with10 µg Naked pDNA 112
6.3.2 Haemagglutination Inhibition Assay 113
6.3.3 Characterization of Dextran-DNA nanoparticles 114
6.3.4 Antibody response after *in ovo* immunization with Naked pDNA and pDNA/Dextran-spermine 118
6.3.5 HI Assay Analysis 124
6.3.6 Result of Post Challenge with NDV AF2240 and IBS002 Strains 125
6.4 Conclusion 130

7 DISCUSSION, CONCLUSION AND FUTURE RECOMMENDATIONS 132

REFERENCES/BIBLIOGRAPHY 145
APPENDICES 164
BIODATA OF STUDENT 185
LIST OF PUBLICATIONS 186