



UNIVERSITI PUTRA MALAYSIA

**TOWARDS THE DEVELOPMENT OF DNA VACCINE AGAINST
NEWCASTLE DISEASE VIRUS**

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By

LOKE CHUI FUNG

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The etiological agent of Newcastle disease, Newcastle disease virus (NDV), a member of the family *Paramyxoviridae* and the genus of *Rubulavirus*, can cause up to 100% morbidity and mortality. Immune responses to both the fusion (F) and haemagglutinin-neuraminidase (HN) protein antigens of NDV were demonstrated to play an important role in the prevention of infection. Towards development of DNA vaccine, both the F and HN genes of a Malaysian heat resistant viscerotropic-velogenic NDV strain AF2240 were amplified and cloned into a mammalian expression vector, pEGFP-Ns, and expressed in a mammalian cell line under the control of the immediate early promoter of human cytomegalovirus.

Six recombinant plasmids were constructed, namely pEGFP-N3/F, -N1/HN, -N3/HN-GFP, -N2/Fkoz, -N2/HNkoz and -N1/Fkoz-GFP with the later three constructs introduced with the *kozak* translation initiation sequences. Transient expression of F and HN proteins was assayed in vitro in Vero cell at 48 h post-transfection by indirect immunofluorescence using NDV polyclonal antibody and

fluorescein isothiocyanate (FITC)-labelled anti-chicken IgG. The results 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. Immunoblot analysis of the transfected cell lysates further verified the presence of the recombinant proteins with a distinct band of 64 kDa which corresponds to the uncleaved precursor F₀ glycoprotein of NDV and two bands of ~62 and 72 kDa as unglycosylated and glycosylated HN glycoproteins, respectively. [³⁵S]-methionine pulsed labelling of transfected cells confirmed the expression of green fluorescent protein (GFP)-fusion protein of F, but not HN-GFP.

DNA inoculation in Balb/c mice and specific pathogen free (SPF) chicken revealed that the efficacy of DNA vaccines could be boosted by co-administration of Freund's adjuvant and repeating DNA immunization. The vaccine trial in SPF chickens showed that both the circular and linearized plasmid DNA of pEGFP-N3/F produced significant levels of antibody against NDV after the second booster and conferred 40-47% protection upon lethal NDV challenge. Co-administration of the circular plasmids of pEGFP-N3/F and -N1/HN, produced antibodies efficiently and conferred more than 50% protection upon NDV challenge. The low and undetectable antibody level in some of the survivors suggests that DNA vaccine elicits cellular immune response in chicken. The overall results also suggest that both the F and HN-DNA can be used as a vaccine component to provide effective protection against NDV and DNA immunization opens a new approach to the development of gene vaccine for chicken against infectious disease.



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MENUJU KE ARAH PERKEMBANGAN VAKSIN DNA UNTUK VIRUS PENYAKIT NEWCASTLE

Oleh

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Agen etiologi penyakit Newcastle, iaitu virus penyakit Newcastle (NDV), anggota famili *Paramyxoviridae* dan genus *Rubulavirus*, boleh menyebabkan 100% morbiditi dan mortaliti. Reaksi keimunan terhadap antigen-antigen protein pertaupan (F) dan hemaglutinin-neuraminidase (HN) NDV memainkan peranan yang penting dalam pencegahan jangkitan. Untuk menghasilkan vaksin DNA, kedua-dua gen F dan HN strain AF2240 NDV Malaysia yang velogenik viserotropik telah diampifikasikan and diklonkan ke dalam vector pengekspresan mamalia, pEGFP-Ns, dan diekspreskan dalam sel mamalia di bawah pengaruh promoter sitomegalovirus manusia.

Enam plasmid rekombinan telah dibina, iaitu pEGFP-N3/F, -N1/HN, -N3/HN-GFP, -N2/Fkoz, -N2/HNkoz dan -N1/Fkoz-GFP dengan ketiga-tiga plasmid terakhir disertakan dengan jujukan permulaan penterjemahan *kozak*. Pengekspresan sementara bagi protein-protein F and HN telah diesèi secara *in vitro* dalam sel Vero 48 jam selepas proses transfeksi oleh imunopendarfluoran dengan menggunakan antibodi poliklonal NDV dan IgG anti-ayam berlabel pendarfluoran isothiosinat (FITC).

Keputusan menunjukkan kesemua sel yang ditranfeksikan DNA memberi pendarfluor sitoplasmik, menandakan kedua-dua protein F dan HN telah diekspreskan dalam sel mamalia. Analisis imunoblot bagi lisat sel transfeksi menunjukkan kehadiran jalur protein rekombinan pada 64 kDa yang berpadan dengan protein prekursor F₀ NDV tidak terpotong dan dua jalur protein pada ~62 dan 72 kDa sebagai HN glikoprotein yang tidak berglikosilasi dan berglikosilasi masing-masing. Sel-sel transfeksi yang berlabel dengan [³⁵S]-methionin menunjukkan pengekspresan protein pendarfluoran hijau (GFP) bertaupan dengan F, tetapi bukan GFP-HN.

Inokulasi DNA dalam tikus Balb/c dan ayam pathogen bebas spesifik (SPF) menunjukkan keefikasian vaksin DNA boleh ditingkatkan dengan penyuntikan bersama adjuvan Freund dan imunisasi DNA berulang. Percubaan vaksin dalam ayam SPF menunjukkan bahawa kedua-dua plasmid DNA bulatan dan terpotong bagi pEGFP-N3/F menghasilkan antibodi setelah suntikan booster kedua and memberi perlindungan 40-47% terhadap saingan NDV. Penyuntikan bersama plasmid-plasmid pEGFP-N3/F dan -N1/HN menghasilkan antibodi yang berkesan dan memberi lebih daripada 50% perlindungan terhadap jangkitan NDV. Tahap antibodi yang terlalu sedikit dalam ayam-ayam yang masih hidup mencadangkan bahawa vaksin DNA dapat menghasilkan tindakbalas keimunan sel dalam ayam. Keputusan keseluruhan juga mencadangkan bahawa kedua-dua DNA F and HN boleh digunakan sebagai komponen vaksin untuk memberi perlindungan efektif terhadap jangkitan NDV dan imunisasi DNA membuka satu pendekatan baru dalam perkembangan vaksin gen untuk ayam daripada dijangkiti penyakit.

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TABLE OF CONTENTS

		Page
ABSTRACT.....		ii
ABSTRAK.....		iv
ACKNOWLEDGEMENTS.....		vi
APPROVAL.....		vii
DECLARATION.....		ix
LIST OF TABLES.....		xiv
LIST OF FIGURES.....		xv
LIST OF ABBREVIATIONS.....		xvii
CHAPTER		
I	INTRODUCTION.....	1
	Background.....	1
	Significance of the Study.....	5
II	LITERATURE REVIEW.....	7
	Newcastle Disease.....	7
	Nature of the Disease.....	7
	Aetiology.....	8
	Pathogenicity.....	10
	World Distribution and Occurrence in Malaysia.....	12
	NDV Strain AF2240.....	13
	The F and HN Genes of NDV Strain AF2240 and Their Encoded Glycoproteins.....	14
	The F Gene and Its Encoded Glycoprotein.....	17
	The HN Gene and Its Encoded Glycoprotein.....	19
	Interaction between the HN and F Proteins.....	20
	The Avian Immune System.....	22
	The Present State of Veterinary Vaccine Development of NDV.....	23
	Live Attenuated NDV Vaccines.....	24
	Killed or Inactivated NDV Vaccines.....	27
	Recombinant Subunit Vaccines of NDV.....	28
	Synthetic Peptide or Polypeptide Vaccines.....	31
	Approaches to the Development of DNA Vaccines against NDV.....	33
	Immune Responses Induced by DNA Immunization.....	34
	Humoral Immune Response.....	35
	Cell-Mediated Immune (CMI) Response.....	36
	CD4 ⁺ T Lymphocytes Function and T-helper Response.....	37

	CD8 ⁺ Cytotoxic T Lymphocyte (CTL) Function and CTL Response.....	39
	Future Prospect and Problem with DNA Vaccines.....	41
III	MATERIALS AND METHODS.....	43
	Molecular Biological Methods.....	43
	General Procedures.....	43
	Newcastle Disease Virus (NDV) Strain AF2240.....	43
	Virus Propagation and Purification.....	44
	Viral RNA Extraction.....	45
	Primer Design.....	46
	Reverse Transcription and Polymerase Chain Reaction (RT-PCR).....	46
	Cloning of HN and F Genes into TOPO pCR™2.1 and Mammalian Expression Vectors.....	48
	Overview of TOPO pCR™2.1 Vector.....	48
	Overview of pEGFP-Ns N-Terminal Protein Fusion Vector.....	49
	Preparation of Competent Cells.....	50
	Unidirectional Cloning and Heat-Shock Transformation.....	51
	TA Cloning.....	52
	Subcloning of Inserts from TOPO pCR™2.1 into pEGFP-Ns.....	53
	Plasmid Extraction and Restriction Enzyme Analysis.....	53
	Nucleotide Cycle Sequencing of F and HN Genes.....	56
	Preparation of Sequencing Gels.....	57
	Cell Culture Methods.....	58
	General Procedures.....	58
	Mammalian Cell Culture.....	59
	Overview of Vero Cells.....	59
	Maintenance of Vero Cell Line.....	59
	Leibovitz's L-15 Medium.....	59
	Cultivation of Vero Cells from Frozen Stock Culture.....	60
	Preparation of Stock Culture.....	61
	Transient Transfection of Adherent Mammalian Cells... Overview of LIPOFECTAMINE™ Reagent.....	61
	Transient Cationic Lipid Transfection of Adherent Mammalian Cells.....	62
	Stable Cationic Lipid Transfection Using Geneticin® (G418).....	63
	Determination of Toxic Concentration of Geneticin® (G418).....	63
	Selection of Transfected Cells.....	63
	PCR Analysis of Transfected Cells.....	64
	Identification of the Recombinant F and HN Glycoproteins.....	65

	Indirect Immunofluorescence.....	65
	SDS-Polyacrylamide Gels	
	Electrophoresis (SDS-PAGE).....	66
	Preparation of SDS-	
	Polyacrylamide Gels.....	66
	Sample preparation and SDS-	
	PAGE.....	66
	Immunoblotting.....	67
	[³⁵ S]-Methionine Pulsed Labeling of	
	Transfected Cells.....	68
	DNA Immunization Studies in Animal Models.....	69
	DNA Immunization in Balb/c Mice.....	69
	Enzyme Link Immunoabsorbent Assay (ELISA)	
	Using NDV Strain AF2240 Coated Plate.....	72
	Extraction of Genomic DNA from Mammalian	
	Tissue.....	72
	Extraction of mRNA from Mammalian	
	Tissue.....	73
	mRNA-RT-PCR Analysis.....	75
	DNA Immunization in Specific-Pathogen-Free	
	(SPF) Chickens.....	75
	DNA Immunization Regimen I.....	75
	DNA Immunization Regimen II.....	77
	Viral Challenge.....	77
	ELISA.....	78
	ELISA using NDV Strain AF2240 Coated Plate.	79
	Haemagglutinin Inhibition (HI) Assay.....	79
IV	RESULTS	80
	Cloning of F and HN Genes into Mammalian Expression	
	Vector.....	80
	Amplification of F and HN Genes of NDV Strain	
	AF2240.....	80
	TA Cloning of RT-PCR F and HN Gene Products into	
	TOPO pCR™ 2.1 Vector.....	81
	Cloning of RT-PCR F and Subcloning of F and HN	
	Inserts Genes from TOPO pCR™ 2.1 Vector into	
	pEGFP-Ns.....	84
	Verification of Putative Recombinant F and HN Clones	
	in pEFGP-N Vectors.....	85
	Partial End Terminal Sequencing of the Putative F and	
	HN Recombinant Plasmids.....	87
	Common Morphology of Vero Cell Cultures.....	87
	Optimisation of Transfection Efficiency with Parental Plasmid	
	Vector.....	91
	Transient Expression of F and HN Genes in a Mammalian Cell	
	Line.....	92
	Detection of Transfection Cells.....	92
	Indirect Immunofluorescence (IIF).....	94

SDS-PAGE and Western Blot Analysis.....	97
Dectection of F- and HN-GFP Fusion Proteins.....	100
Fluorescence Microscopy.....	100
Immunoprecipitation.....	100
[³⁵ S]-Methionine Labelling and Western Blot Analysis.....	102
Immunogencity Evaluation of DNA Vaccine.....	104
DNA Immunization of Balb/c Mice.....	104
Induction of Humoral Responses after DNA Immunization.....	104
Longevity of Responses.....	107
Detection of Transgenes and Duration of Transgene Expression in Mice.....	109
DNA Immunization in SPF Chickens.....	109
1 st Immunization Regimen.....	112
Effect of Adjuvants in DNA Immunization.....	112
Antibody Responses Following Coadministration of F and HN Plasmid Constructs.....	115
Viral Challenge.....	115
Degree of Severity of Viral Infection after Viral Challenge.....	118
2 nd Immunization Regimen.....	119
V GENERAL DISCUSSION.....	126
<i>In Vitro</i> Studies.....	126
<i>In Vivo</i> Studies.....	131
DNA Immunization in Balb/c Mice.....	131
DNA Immunization in SPF Chickens.....	135
Humoral Responses in Vaccinated SPF Chickens.....	135
1 st Immunization Regimen.....	135
2 nd Immunization Regimen.....	137
Overall Discussion.....	139
VI CONCLUSION AND FUTURE RECOMMENDATIONS.....	149
BIBLIOGRAPHY.....	152
APPENDICES.....	176
Appendix A.....	176
Appendix B.....	182
Appendix C.....	187
Appendix D.....	209
BIODATA OF THE AUTHOR.....	210

LIST OF TABLES

Table		Page
1	Oligonucleotide Primers Used in Amplification of Various F and HN Open Reading Frames.....	47
2	Clones Used in the Study.....	54
3	Plasmid DNAs Used for Immunization in Balb/c Mice.....	70
4	Plasmid DNAs Used for Immunization in SPF Chickens.....	76
5	Details of Recombinant Clones Used in the Study.....	86
6	PCR and RT-PCR Analysis of Genomic DNA and mRNA Extracted from Various Tissues of Immunized Mice.....	110
7a	ELISA and HI Titers for Vaccinated Birds (Regimen I: without Adjuvant).....	113
7b	ELISA and HI Titers for Vaccinated Birds (Regimen I: with Adjuvant)	114
8	Degree of Severity of Viral Infection in All Dead Birds (Regimen I).....	120
9	Antibody and HI Titers for Vaccinated Birds (Regimen II).....	122

LIST OF FIGURES

Figure		Page
1	Electron Micrograph of NDV Strain AF2240.....	9
2	Nucleotide Sequence of F Gene of NDV Strain AF2240.....	15
3	Nucleotide Sequence of HN Gene of NDV Strain AF2240.....	16
4	Purified Amplified RT-PCT Products Containing the F and HN Gene Inserts.....	82
5	End terminal Sequencing on Positive F Clones.....	88
6	End terminal Sequencing on Positive HN Clones.....	89
7	Morphology of Vero Cells.....	90
8	Efficiency of Different Transfection Parameters on the Expression of Green Fluorescent Protein (GFP).....	93
9	Amplification Analysis of Transfected Vero Cells.....	95
10	Transient Transfection of Vero Cells with Plasmids, pEGFP-N2/Fkoz, -N2/HNkoz, -N3/F and -N1/HN.....	96
11	SDS-PAGE and Western Blot Analysis of Vero Cells Transfected with Recombinant Plasmids, pEGFP-N2/ Fkoz and -N2/HNkoz.....	98
12	SDS-PAGE and Western Blot Analysis of Vero Cells Transfected with Recombinant Plasmids, pEGFP-N3/F, -N1/HN, -N3/HN/GFP and -N1/Fkoz-GFP.....	99
13	Detection of GFP Fusion Proteins by Fluorescence Microscopy.....	101
14	Immunoprecipitation of F- and HN-GFP Fusion Proteins from Transfected Vero Cells.....	103
15	Detection of F- and HN-GFP Fusion Proteins by [³⁵ S]-Methionine Labelling.....	105
16	Kinetics of Total anti-NDV Antibodies in Mice Immunized with Different Plasmid Constructs.....	106
17	Comparison of Antibody Responses Elicited in Balb/c Mice by Immunization of Indicated Plasmid Constructs.....	108

18	RT-PCR Analysis of mRNA Extracted from Various Organs of Mice	111
19	Comparison of Antibody Responses Elicited in SPF Chickens Following Vaccinations with Indicated Plasmid Constructs Emulsified with and without Adjuvant Using ProFLOK NDV ELISA Kit (KPL, USA).....	116
20	Comparison of Antibody Responses Elicited in SPF Chickens Following Vaccinations with Indicated Plasmid Constructs Emulsified with and without Adjuvant Using NDV AF2240 Coated ELISA Plate.....	117
21	Antibody Responses of Chickens Injected with Respective DNA Constructs.....	124

LIST OF ABBREVIATIONS

A₂₆₀	-	absorbance at 260 nm
Ag	-	antigen
APCs	-	antigen presenting cells
APS	-	ammonium persulfate
bp	-	base pair
BCP	-	1-bromo-3-chloro-propane
BSA	-	bovine serum albumin
cDNA	-	complementary deoxyribonucleic acid
Ci	-	curie
CMI	-	cell-mediated immune
CTL	-	cytotoxic T lymphocyte
dATP	-	deoxyadenosine triphosphate
dCTP	-	deoxycytidine triphosphate
ddATP	-	dideoxyadenosine triphosphate
ddCTP	-	dideoxycytidine triphosphate
ddGTP	-	dideoxyguanosine triphosphate
ddNTP	-	dideoxynucleotide triphosphate
ddTTP	-	dideoxythymidine triphosphate
DEPC	-	diethylpyrocarbonate
dGTP	-	deoxyguanosine triphosphate
DMSO	-	dimethyl sulfoxide
DNA	-	deoxyribonucleic acid
DNase	-	deoxyribonuclease

dNTP	-	deoxynucleotide triphosphate
DTT	-	dithiothreitol
dTTP	-	deoxythymidine triphosphate
EDTA	-	ethylenediaminetetraacetic acid disodium salt
EGFP	-	enhanced green fluorescent protein
ELISA	-	enzyme-linked immunosorbent assay
F	-	fusion gene/protein
FITC	-	fluorescein isothiocyanate
<i>g</i>	-	gravity
h	-	hour
HI	-	haemagglutination inhibition
HN	-	haemagglutinin-neuraminidase
HRP	-	horseradish peroxidase
IE	-	immediate early
IFN	-	interferon
Ig	-	immunoglobulin
IL	-	interleukin
kb	-	kilobase
kDa	-	kilodalton
Mab	-	monoclonal antibody
MCS	-	multiple cloning site
MHC	-	major histocompatibility complex
Mr	-	relative molecular weight
mRNA	-	messenger ribonucleic acid
NDV	-	Newcastle disease virus

OD	-	optical density
ORF	-	open reading frame
PBS	-	phosphate-buffered saline
P _{CMV}	-	cytomegalovirus promoter
PCR	-	polymerase chain reaction
PVDF	-	polyvinylidene difluoride
RBC	-	red blood cell
RNA	-	ribonucleic acid
RNase	-	ribonuclease
RT-PCR	-	reverse transcriptase-polymerase chain reaction
s	-	second
SDS-PAGE	-	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIg A	-	secretory immunoglobulin A
SPF	-	specific pathogen free
SV40	-	simian virus 40
Taq	-	<i>Thermus aquaticus</i>
TBE	-	Tris-boric-EDTA buffer
TCR	-	T cell receptor
TEMED	-	<i>N,N,N',N'</i> , -tetramethylethylenediamine
Th	-	T helper
Tris-HCl	-	Tris hydrochloride
UV	-	ultraviolet
X-gal	-	5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside

CHAPTER I

INTRODUCTION

Background

Newcastle disease (ND) continues to be one of the most important constraints to the development of improved livestock production in both developing and developed countries (Awan *et al.*, 1994; Maldonado *et al.*, 1994; Alexander, 1995). At present, animal husbandry and production industries, particularly in the developing countries, are attempting to meet the demands for increased food production from expanding human populations. In Peninsular Malaysia, the poultry industry has developed to a most scientifically advanced industry and contributes more than 60% of the total value of livestock. In order to maintain and increase the productivity of poultry products, both the vaccination and therapeutic or prophylactic use of drugs play an important role in animal disease control. Now, vaccination is increasingly being viewed as the most sustainable option, as it has a major impact on the control of epidemic viral disease of livestock such as foot-and-mouth disease (Bachrach *et al.*, 1975; Di Marchi *et al.*, 1986) and rinderpest (Romero *et al.*, 1993). Moreover, it has the potential to offer for greater economic efficiency in every aspect.

Vaccination against ND virus (NDV) is a common practice worldwide in the poultry industry (Beard and Hanson, 1984; Glisson and Kleven, 1993). In the past

decades, protection from ND has traditionally relied on the use of either attenuated or killed vaccines. The most widely used vaccines are live viruses consisting of either lentogenic or selected mesogenic strains propagated in embryonated chicken eggs. The most common NDV vaccine strains are V4, Ulster 2C, Hitchner B1, Asplin F and La Sota (all lentogenic) and Roakin, Mukteswar and Komarov (all mesogenic) (reviewed in Alexander, 1988). Both the attenuated and killed vaccines have been used successfully to induce protective level of immunity among the poultry not only in Malaysia, but also in Australia and many other parts of the world where endemic NDV is prevalent (Alexander, 1995). Nonetheless, problems may arise in field when insufficient attention is paid to factors such as age of the birds, route of inoculation, the strains of virus and follow-up serology (Taylor *et al.*, 1990; Glisson and Kleven, 1993). For live attenuated vaccine, there is always a degree of unpredictability as it may cause clinical disease if not attenuated sufficiently (Mckee *et al.*, 1987) and most importantly, the possibility of reversion to a more virulent potentially disease-causing phenotype which can establish persistent or latent infection to the host (Weeks-Levy *et al.*, 1991; Ogra *et al.*, 1991). In addition, live recombinant vaccine, which is engineered to express recombinant genes of which its products are immunogenic, is able to induce both humoral and cell-mediated responses, however, it may not be safe for immunocompromised individuals such as AIDS patients. On the other hand, killed vaccines are generally safe, but unable to generate protective levels of immunity for reasons of losing important epitopes of antigen during inactivation (reviewed in Cox *et al.*, 1993). Moreover, multiple boosters are usually necessary in order to generate continual antigen exposure. Furthermore, killed vaccines do not sustain in the host or enter into the host cell, therefore, do not synthesize endogenous protein and unable to

induce cytotoxic T cells (Monaco, 1992), possibly a desirable property of an effective vaccine (Leung and Ada, 1982; Taylor and Askonas, 1986). In addition, killed vaccines also have potential risk in which incomplete or improper killing of virus could result in the contamination of vaccines with active wild type virus, especially during large-scale production of the virus (reviewed in Kang, 1989).

In the early 1980s, the advent of recombinant DNA technology created excitement and provided new opportunities to produce vaccines based on the use of expressed products of cloned genes. The approach of using purified recombinant proteins or subunit protein vaccines, consisting of non-replicating and non-infectious portions of the pathogenic agent, has provided some solutions to problems such as incomplete inactivation, unsatisfactory attenuation of the virus and the possible biological contamination of the vaccines that may occur during large-scale production of virus. The use of purified recombinant proteins for vaccination has shown success where the immunity has been achieved with antigen produced in *Escherichia coli*, yeast or other eukaryotic systems (McAleer *et al.*, 1984; Willadsen *et al.*, 1989; Musoke *et al.*, 1992). Recently, the advent of expression vector such as baculoviruses, which can produce large quantities of desired proteins in cell culture, has greatly enhanced the possibilities for other subunit vaccine development (Miller, 1988; Marshall and Roy, 1990; Pearson and Roy, 1993). Undoubtedly, subunit protein vaccines are safe to use. However, they are easily denatured during purification and are not always in their native form when introduced into the host (Finn, 1998), hence, making them poorly immunogenic.

Despite the effectiveness of the current vaccines, many such vaccines are still inadequate for the reasons of safety, cost effectiveness and efficacy related to the risk of infection from the emergence of vaccine-escape mutant (Carman *et al.*, 1990). Therefore, further development of new vaccines to improve the efficacy of vaccination is desirable. At present, direct gene transfer into mammalian somatic tissue *in vivo* is a powerful approach for gene therapy with potential application. Within a decade, the field of genetic vaccination has been studied and developed rapidly and has taken on a new urgency as the vulnerability of related populations to infectious disease increases. The development of DNA vaccine against viral infection has become a great surprise and challenge in the molecular medicine after its first introduction by Wolff *et al.* in 1990 as DNA vaccines can induce both humoral and cell-mediated immunity. Since then, immunization by direct injection of DNA has been working out towards the induction of protective antiviral immunity in the animal models (Ulmer *et al.*, 1993; Yokoyama *et al.*, 1995; Armas *et al.*, 1996; Sakaguchi *et al.*, 1996; Chow *et al.*, 1997).

Direct injection of DNA into animals can result in immune responses to protein encoded by DNA. Although this vaccination strategy is relatively new, it has already been shown to be successful in generating protective immunity against influenza virus (Ulmer *et al.*, 1993; Larsen *et al.*, 1998), rotavirus (Herrmann *et al.*, 1996), lymphocytic choriomeningitis virus (Pedroza *et al.*, 1995; Yokoyama *et al.*, 1995), herpes simplex virus (Manickan *et al.*, 1995; Bourne *et al.*, 1996; Mangala *et al.*, 1998) and hepatitis B virus (Triyatni *et al.*, 1998). Like live-virus vaccines, DNA vaccines can induce both antibody and T-cell responses. The latter comprises both

major histocompatibility complex (MHC) class II-restricted CD4⁺ T cells, most often of helper phenotype and MHC class I-restricted CD8⁺ cytotoxic T-lymphocytes (CTLs), which kill or lyse infected cells by means of the action of protein perforin released by CD8⁺ CTLs (reviewed in Griffiths and Tschopp, 1995). CD8⁺ CTLs have been demonstrated to be a major host defence mechanism against many viruses since the last two decades (Yap *et al.*, 1978; Buchmeier *et al.*, 1980; Lin and Askonas, 1981; Kast *et al.*, 1986). However, the importance of CTL response induced by DNA vaccine was only highlighted recently (Doe *et al.*, 1996; Iwasaki *et al.*, 1997a; Ulmer *et al.*, 1998). At present, DNA-based immunization has yet been applied to human commercially, however, clinical trials on human have been underway (Finn, 1998; Wang *et al.*, 1998). There are many potential problems related to the use of DNA vaccines. For example, the expression of vector derived antigens may negatively influence the specific immune response in the host; the possibility of insertional mutagenesis that occur may lead to the unpredictable side effects such as switching on or off of oncogenes and tumor suppressor genes (reviewed in McDonnell and Askari, 1996). Therefore, the safety of using DNA vaccine remains an issue. Still, DNA vaccination is likely to become a valuable tool for diverse types of therapeutic interventions.

Significance of the Study

In the present study, we intended to develop DNA vaccines against the Malaysian heat resistant viscerotropic-velogenic Newcastle disease virus (NDV) strain AF2240 with both the fusion (F) and haemagglutinin-neuraminidase (HN)