UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF GENE DELETED RECOMBINANT PSEUDORABIES VIRUS

ZEENATHUL NAZARIAH BT ALLAUDIN

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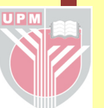

DEVELOPMENT OF GENE DELETED RECOMBINANT PSEUDORABIES VIRUS

By

ZEENATHUL NAZARIAH BT ALLAUDIN

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

August 2004

DEDICATION

DEDICATED WITH LOVE AND GRATITUDE TO:

MY HUSBAND, CHILDREN,

PARENTS,

BROTHERS AND SISTERS.

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

DEVELOPMENT OF GENE DELETED RECOMBINANT PSEUDORABIES VIRUS

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August 2004

Chairman: Professor Mohd. Azmi Mohd. Lila, Ph.D.

Faculty: Veterinary Medicine

The glycoprotein E (gE) and thymidine kinase (TK) genes are virulence-associated genes of pseudorabies virus (PrV). The study conducted was to shut down the gE gene from an established local TK defective (TK^-) PrV strain ($TK^- gE^+$PrV). The ultimate aim of the study was to develop a gene-deleted recombinant PrV with useful identification markers.

A gE gene-deleted pseudorabies virus ($TK^- gE^-$ PrV) was constructed by homologous recombinational techniques. The $TK^- gE^+$PrV, regarded as the parental strain in the study, originated from a virulent local PrV isolate ($TK^+ gE^+$PrV). Prior to the construction of the $TK^- gE^-$ PrV, the gE of the parental strain was amplified, cloned and studied. Comparative sequence analysis showed that the gE sequence of $TK^- gE^+$PrV was closely identical (98 %) to a Chinese Ea strain. The 10 nucleotide variations at nucleotide positions 237, 931, 1207, 1409, 1501, 1530, 1549, 1555, 1682 and 1842, led to six amino acids substitutions at amino acid residues 403 (A ▾ P), 470 (V ▾ A), 501 (V ▾ I), 517 (P ▾ S), 519 (T ▾ A) and 561 (T ▾ N) in their open reading frames (ORFs) that code a 578 amino acid polypeptide. All 10 cystein

clusters in the gE sequences of the PrV strains namely $TK^{-}gE^{+}$ PrV, Ea strain and Rice strain were conserved. Despite the low overall level of amino acid sequence identity among the gE proteins (23 to 31%) of diverse animal species, the cystein clusters were relatively well conserved especially in the C-terminal of the protein. The 500 bp deletion introduced into $TK^{-}gE^{+}$ PrV gE gene, was designed to remove three cystein residues and one potential N-glycosylation site at the C-domain of gE, while maintaining sufficient flanking regions within the gE gene to facilitate homologous recombination.

The $TK^{-}gE^{-}$ PrV constructed was identified by gene specific PCR assay, gE-PCR profiles and sequence analysis. Expression analysis by SDS-PAGE and immunoblots proved the absence of gE protein. The absence of gE-specific antibodies in the serum of $TK^{-}gE^{-}$ PrV immunized murine models further substantiated the evidence. Besides, the protective nature of $TK^{-}gE^{-}$ PrV resembled that of parental strain ($TK^{-}gE^{+}$PrV). Based on the gE deletion site, $TK^{-}gE^{-}$ PrV can be clearly differentiated from other PrV vaccine strains. Overall, the gE deletion was proven to be a functional genetic cum serologic marker.

It is intriguing to know whether the virus is useful to deliver and express a foreign gene within the gE expression locus. Therefore, an E2 gene expression cassette, originally from classical swine fever (CSFV), was specially designed to be incorporated into the deleted gE gene as a foreign insert. It serves as a CSFV marker as well as for its immunogenic and protective properties against CSFV infection. An eukaryotic expression vector was constructed to express the CSFV E2 gene with specific functional domains. Following transfection of mammalian cells with the E2

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encoded naked plasmid (pCDNA+E2), E2 protein was detected using immunoperoxidase staining, SDS-PAGE and immunoblot analyses. Before the gene was introduced into the gE gene of PrV, the ability of the expression plasmids to induce immune response *in vivo* was also evaluated in mice via gene gun and intramuscular injections. Both humoral and cell-mediated immunity were detected. Therefore, the CSFV E2 expression cassette developed was determined to be appropriate for a recombinant with $TK^{-} gE^{-}$ PrV.

The recombinant PrV was successfully developed, primarily by genetically modifying the transfer plasmid. The pUC plasmid was manipulated and constructed to harbor the E2 expression cassette with flanking PrV gE nucleotide regions to facilitate homologous recombinant. The construct was transferred into $TK^{-} gE^{-}$ PrV genome by homologous crossovers DNA recombination. The expression of the E2 gene in a viral plaque indicated a successful integration of the gene in PrV genome. The formation of designated $TK^{-} gE^{-} E2^{+}$PrV virus particles were verified by means of PCR and sequence analysis.

Based on its characteristics, generally it is concluded that the gene deleted pseudorabies virus, $TK^{-} gE^{-}$PrV, is a good candidate for preparation of an attenuated vaccine as well as a viral vector.

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

PERKEMBANGAN VIRUS PSEUDORABIES REKOMBINAN TERHAPUS GEN

Oleh

ZEENATHUL NAZARIAH BT ALLAUDIN

Ogos 2004

Pengerusi: **Profesor Mohd. Azmi Mohd. Lila, Ph.D.**

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Gen glikoprotein E (gE) dan timidina kinase (TK) merupakan gen terkait virulens virus pseudorabies (PrV). Kajian ini telah dijalankan untuk mengancing gen gE daripada suatu strain tempatan PrV ($TK^{-}gE^{+}PrV$) cacat TK (TK^{-}) yang tertubuh. Matlamat akhir kajian ini ialah untuk mengembangkan PrV rekombinan terhapus gen yang mempunyai penanda pengenalpastian berguna.

Virus pseudorabies terhapus gen gE ($TK^{-}gE^{+}PrV$) telah dibentuk melalui teknik rekombinasi homologus. $TK^{-}gE^{+}PrV$ yang dianggap sebagai strain induk dalam kajian ini, adalah berasal daripada pencilan PrV tempatan virulen ($TK^{+}gE^{+}PrV$). Sebelum pembentukan $TK^{-}gE^{-}PrV$ ini, gE daripada strain induk terlebih dahulu diamplifikasikan, diklon dan dikaji. Analisis jujukan bandingan menunjukkan yang jujukan gE $TK^{-}gE^{+}PrV$ adalah bersecaman rapat (98%) dengan strain Ea Cina. Ada 10 perbezaan nukleotida pada kedudukan 237, 931, 1207, 1409, 1501, 1549, 1555, 1682, dan 1842, yang membawa kepada penggantian enam asid amino pada residu asid amino 403 (A ▼ P), 470 (V ▼ A), 501 (V ▼ I), 517 (P ▼ S), 519 (T ▼ A) dan 561 (T ▼ N) pada rangka bacaan terbukanya (ORF) yang mengekodkan suatu

polipeptida 578 asid amino. Kesemua 10 gugusan sisteina dalam jujukan gE strain PrV, iaitu $TK^{-} gE^{+}PrV$, EA dan Rice masih terpelihara. Walaupun aras sepercaman keseluruhan jujukan asid amino di kalangan protein gE pelbagai spesies haiwan itu rendah (23 hingga 31%), gugusan sisteina adalah agak terpelihara dengan baik pada C-penghujung protein. Penghapusan 500 kb yang diperkenalkan kepada gen gE $TK^{-} gE^{+}PrV$, adalah direka bentuk untuk mengeluarkan tiga residu sisteina dan satu tapak berpotensi N-pengglikosilan dalam C-domain gE, sambil mengekalkan secukupnya kawasan sisi dalam gen gE untuk memudahkan rekombinasi homologus.

$TK^{-} gE^{-} PrV$ yang dibentuk itu dikenal pasti melalui assai PCR khusus gen, profil gE-PCR, dan analisis jujukan. Analisis penyataan melalui SDS-PAGE dan imunosap telah mengesahkan ketiadaan protein gE. Ketiadaan antibodi gE-khusus dalam serum model murin terimun $TK^{-} gE^{-} PrV$ telah menyokong bukti ini. Tambahan pula sifat pelindung $TK^{-} gE^{-} PrV$ menyerupai strain induk ($TK^{-} gE^{+}PrV$). Berasaskan tapak penghapusan gE, $TK^{-} gE^{-} PrV$ secara jelas boleh dibezakan daripada strain vaksin PrV lain. Secara keseluruhan, penghapusan gE ini membuktikan yang ianya adalah penanda genetik dan serologi berfungsi.

Perkara yang menarik perhatian ialah sama ada virus ini berguna untuk pembawaan dan penyataan suatu gen asing dalam lokus penyataan gE. Oleh itu, satu kaset penyataan gen E2, yang asalnya daripada virus demam babi klasik (CSFV), telah direka bentuk khusus untuk dimasukkan ke dalam gen gE terhapus sebagai suatu selitan asing. Ianya bertindak sebagai penanda CSFV dan juga dipilih kerana sifat imunogenik dan perlindungannya terhadap jangkitan CSFV. Satu vektor penyataan eukariot telah dibentuk untuk menyatakan gen E2 CSFV dengan domain fungsian khusus. Berikutan transjangkitan sel mamalia dengan plasmid yang mengekodkan gen E2 (pCDNA+E2), protein E2 dikesan melalui pewarnaan imunoperoksidase,

SDS-PAGE, dan analisis imunosap. Sebelum gen diperkenalkan kepada gen gE PrV, keupayaan plasmid penyataan untuk mengaruh gerak balas imun *in vivo* dinilai juga dalam mencit melalui penembak gen dan suntikan intraotot. Kedua-dua keimunan humoral dan berantarakan sel telah dikesan. Dengan ini, kaset penyataan E2CSFV yang dikembangkan itu telah dipastikan sesuai untuk rekombinasi dengan TK^{-} gE^{-} PrV.

PrV rekombinan telah berjaya dikembangkan, khususnya melalui pengubahsuaian genetik plasmid pemindah. Plasmid pUC telah dimanipulasi dan dibentuk untuk mengandungi kaset penyataan E2 dengan kawasan nukleotida gE PrV pada sisinya untuk memudahkan rekombinasi homologus. Struktur ini telah dipindah ke dalam genom TK^{-} gE^{-} PrV melalui rekombinasi DNA lintas homologus. Penyataan gen E2 dalam plak virus menunjukkan integrasi gen dalam genom PrV telah bejaya dilakukan. Pembentukan zarah virus TK^{-} gE^{-} $E2^{+}$PrV matlamat telah disah betul melalui PCR dan analisis jujukan.

Berasaskan cirinya, secara umum kesimpulannya ialah, virus pseudorabies terhapus gen, TK^{-} gE^{-} PrV merupakan calon yang baik untuk penyediaan vaksin teratenuat dan sebagai vektor virus.

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I certify that an Examination Committee met on 2nd of August, 2004 to conduct the final examination of Zeenathul Nazariah bt Allaudin on her Doctor of Philosophy thesis entitled "Development of Gene Deleted and Recombinant Pseudorabies Viruses" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

____

ZEENATHUL NAZARIAH BT ALLAUDIN

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LIST OF ABBREVIATIONS

aa	Amino acid
ABTS	2’2-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid)
Ab	Antibody
Ad	Adenovirus
AHV	African horsesickness virus
APC	Antigen presenting cells
ATV	Antibiotic trypsin versine
BHV	Bovine herpesvirus
bp	Base pair
BSA	Bovine serum albumin
BUK	Bucharest strain of pseudorabies
BVD	Bovine viral diarrhoea
BVDV	Bovine viral diarrhoea virus
C protein	Nonglycosylated nucleocapsid protein
cDNA	Complementary deoxyribonucleic acid
CEF	Chicken embryo fibroblast
CHV	Canine herpesvirus
CMI	Cell mediated immunity
CMV	Cytomegalovirus
CNS	Central nervous system
CO_2	Carbondioxide
CPB	Citrate-phosphate buffer
CPE	Cytopathic effect
CRPV	Cotton-tail rabbit papillomavirus
CSF	Classical swine fever
CSFV	Classical swine fever virus
C-terminal	COOH-terminal
CTL	Cytotoxic T-lymphocytes
CVV	Crystal violet vaccine
DAB	3’-3’-diaminobenzidine-hydrochloride
DEPC	Diethyl pyrocarbonate
DIVA	Differentiating infected from vaccinated animals
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTH	Delayed type hypersensitivity
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetate
EHV	Equine herpesvirus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic-reticulum
Fab	Antigen binding fragment
FAO	Food and Agriculture Organization
Fc	Crystallizable fragment
FC	Final concentration
FCS	Foetal calf serum

FHV	Feline herpesvirus
FMDV	Foot and mouth disease virus
g.g.	Gene gun
GaHV	Gallid herpesvirus
gE	Glycoprotein E
GPK	Guinea pig kidney cells
H	Haemagglutinin
H_2O_2	Hydrogen peroxide
hCMV	Human cytomegalovirus
HIS	Hyperimmune serum
HIV	Human immunodeficiency virus
hr	Hour
HR	Homologous recombination
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
i.d.	Intradermal
i.m.	Intramuscular
IgG	Immunoglobulin G
IL	Interleukin
IPTG	Isopropylthiogalactosidase
IR	Inverted repeat sequence
kb	Kilobase pair
kDa	Kilodalton
LB	Lennox L medium
M	Molar
m.o.i	Multitude of infection
MAb	Monoclonal antibody
MDV	Marek's disease virus
MEM	Minimum essential media
MHC	Major histocompatibility complex
min	minute
ml	Mililitre
mm	Milimetre
mM	Milimolar
mRNA	Messenger ribonucleic acid
MVP	Malaysian Vaccine and Pharmaceuticals, Puchong, Malaysia
NCBI	National Center for Biotechnology Information
NDV	Newcastle disease virus
ng	nanogramme
nm	nanometer
NS3	Non-structural protein 3; conserved among pestiviruses
N-terminal	H_2N-terminal
OD	Optical density
OIE	Office International des Epizooties
ORF	Open reading frame
PBS	Phosphate-buffer saline
PBST	Phosphate-buffer saline tween 20
PCR	Polymerase chain reaction
PFU	Plaque forming unit
PRRSV	Porcine reproductive and respiratory syndrome virus

PrV	Pseudorabies virus
PVDF	Polyvinylidene fluoride membrane
RE	Restriction endonuclease
RNA	Ribonucleic acid
RR	Ribonucleotide reductase
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
RV	Rabies virus
SAPS	Statistical analysis of protein sequence
SAPS	Statistical analysis of protein sequence
SDS	Sodium dodecyl sulfate
SDSC	San Diego Supercomputer Center
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Second
SHV	Simian herpesvirus
SPF	Specific pathogen free
STE	Swine testicle
SVV	Simian varicella virus
TA	Tibialis anterior muscle
TE	Tris-EDTA
TGN	Trans-golgi network
Th	T-lymphocyte helper
TK	Thymidine kinase
TK^{-} gE^{-} PrV	Pseudorabies virus with defective in TK and gE genes
TK^{-} gE^{-} E2^{+} PrV	An E2 gene (of CSFV) recombinant pseudorabies virus with defective TK and gE PrV genes
TK^{-} gE^{+} PrV	Pseudorabies virus with defective TK gene
TK^{+}gE^{+} PrV	Wild type pseudorabies
TMAP	Prediction of transmembrane segments
TMR	Transmembrane region
TNE	Tris-NaCl-EDTA
TR	Terminal repeat
U	Unit
UL	Unique Long Sequence
US	Unique Short Sequence
UTR	Untranslated Region
UV	Ultraviolet
μ	micron
μg	microgramme
v/v	volume/volume
Vero	African green monkey kidney cell
VZV	Varicella zoster virus
w/v	Weight/volume
WEE	Western equine encephalitis
xg	gravity
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

LIST OF NOTATION

The names of the 20 common amino acids and their corresponding three-letter abbreviation and single letter code.

Amino acid	Three-letter code	Single-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V