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DEVELOPMENT OF IRES-INCORPORATED LACTOCOCCAL BICISTRONIC VECTOR FOR GENE DELIVERY INTO EUKARYOTIC CELLS

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By

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February 2016

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Internal ribosome entry sites (IRES) are viral RNA elements associated with the construction of bicistronic or polycistronic vectors for the expression of multiple genes in an eukaryotic system due to its ability to initiate translation of mRNA internally. Currently, lactic acid bacteria (LAB) and their vectors have been garnering attention for their prospective applications similar to their *E. coli* counterparts. However, lack of available lactococcal vectors, especially for heterologous protein production in eukaryotes is a glaring factor for its underwhelm utilization in research. In addition, available reported lactococcal eukaryotic vector only enabling single gene insertion into the vector, thus allowing only one protein to be produced, although it is a common knowledge that functional proteins from eukaryotes were normally assembled from two or more subunits. The aim of this study focused on the incorporation of IRES and other eukaryotic elements in lactococcal vector, pNZ8048 to drive dual genes expression in eukaryotic cells. The lactococcal-based bicistronic vector was cloned with the VP2 gene of infectious bursal disease virus (IBDV) and a gene encoding green fluorescent protein (GFP) and denoted as pNZ:VIG. The functionality of the bicistronic vector was tested by analyzing co-translation of the VP2 and GFP proteins. The vector was subjected to *in vitro* coupled transcription and translation cell-free system, transient transfection into chicken embryo fibroblast, DF1 cells, and bacteria mediated plasmid transfer using *Lactococcus lactis* as the bacteria carrier. Cell-free lysate extracts incubated with the newly developed vectors showed the presence of 49 kDa and 29 kDa protein bands corresponding to the relative molecular weight (M_r) of VP2 and GFP proteins, respectively, when subjected to SDS PAGE and Western blotting. Transient expression from *in vitro* DNA transfection into DF1 cells was verified with positive detection of green fluorescence emission due to the GFP production and a 49kDa band corresponding to the M_r of the VP2 protein detected from the proteins extracted from transfected DF1 cells. Prior to bactofection, the cell wall of *L. lactis* was weakened with glycine and lysozyme treatment to increase the efficiency of plasmid transferred from *L. lactis* into DF1 cells. Bactofection analysis showed that the plasmid in *L. lactis* can be transferred into DF1 cells and the genes cloned in the plasmid can be transcribed and translated. Protein band relative to the M_r of VP2 protein was detected via SDS PAGE and Western blotting profile and green fluorescence was detected using

fluorescence microscope. The cell wall treatment of *L. lactis* with glycine and lysozyme in this study was shown to increase the plasmid transfer efficiency based on the intensity of the bands detected on the Western blot membrane. Conclusively, the results in this study indicated the ability of the bicistronic lactococcal vector to perform co-expression of two genes delivered via *in vitro* DNA transfection and bactofection thus, making it feasible for expression of other antigenic or therapeutic genes for vaccine and beneficial proteins delivery into eukaryotes.

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

PEMBANGUNAN VEKTOR BISISTRON DIINKORPORASI DENGAN IRES DARI TULANG BELAKANG DNA LAKTOKOKKAL UNTUK PENGHANTARAN GEN KE DALAM SEL EUKARIOT

Oleh

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'Internal ribosome entry sites', IRESs, merupakan elemen virus RNA yang dikaitkan dengan pembinaan vektor DNA bisistron untuk membolehkan lebih dari satu pengekspresan gen dari satu plasmid eukariot. Kini, bakteria laktik asid dan vektor DNA yang diperoleh daripada bakteria ini telah meraih tumpuan dengan pelbagai potensi yang boleh diaplikasikan setanding dengan *E. coli*. Walau bagaimanapun, kekurangan vektor laktokokkal yang ada di pasaran, lebih-lebih lagi untuk penghasilan protein heterolog di dalam eukariot merupakan faktor yang amat jelas dalam kekurangan penggunaannya di dalam penyelidikan. Di samping itu, hanya satu gen yang boleh diklon ke dalam vektor-vektor laktokkal eukariot yang dilaporkan di dalam jurnal penyelidikan. Oleh sebab itu, vektor-vektor tersebut hanya membolehkan satu protein dihasilkan walaupun diketahui umum bahawa kebanyakan protein dari eukariot selalunya dihimpun dari dua atau lebih subunit protein. Kajian ini akan memberi tumpuan ke atas gabungan IRES dan elemen DNA eukariot yang lain ke plasmid laktokokkal, pNZ8048, untuk memandu pengekspresan 2 gen di dalam sistem eukariot. Vektor bisistron yang dibina berasaskan plasmid laktokokkal diklon dengan gen VP2 dari IBDV dan gen pengekodan protein pedarfluor hijau (GFP). Kebolehupayaan vektor bisistron menterjemah gen VP2 dan gen GFP bersama di dalam ekstrak lisat tanpa sel transkripsi dan translasi berganding *in vitro*, transfeksi sementara pNZ:VIG, vektor laktokokkal diinkorporasi dengan IRES yang baru dibina, ke dalam sel fibroblast ayam, DF1 dan interaksi antara sel *L. lactis* membawa vektor yang dibina di dalam kajian ini dengan sel DF1 atau 'bactofection'. Ekstrak lisat tanpa sel dieram bersama vektor DNA yang dibina dan daripadanya analisa SDS PAGE dan pemedapan Western telah dijalankan. Dari profil SDS PAGE dan pemedapan Western, jalur protin 49 kDa dan 29 kDa sepadan dengan jisim molekul relative (M_r) protein VP2 dan GFP telah diperolehi. Di samping itu, keputusan dari transfeksi plasmid DNA ke sel DF1 juga turut diverifikasi dengan pengesanan positif emisi pendarflor hijau dari gen GFP yang diekspresi dan jalur 49 kDa yang sepadan dengan berat molekul protein VP2 daripada protein terkumpul yang diekstrak dari sel DF1 selepas transfeksi. Sebelum menjalankan 'bactofection', dinding sel *L. lactis* telah dirawat dengan glisina dan lisozim untuk melemahkan dinding sel tersebut bagi menambah keberkesanan pemindahan vektor dari sel *L. lactis* ke sel DF1. Analisis dari keputusan 'bactofection'

menunjukkan bahawa plasmid yang telah dibina di dalam *L. lactis* boleh dipindahkan dari *L. lactis* ke sel DF1 dan gen yang diklon di bawah aruhan kaset pengekspresian eukariot boleh diekspresi dari hasil pemedapan Western menandakan kehadiran protein VP2 dari jalur 49 kDa yang dikesan. Rawatan dinding sel *L. lactis* dengan glisina dan lisozim menunjukkan kebolehupayaan pemindahan vektor laktokokkal eukariot dari sel *L. lactis* ke sel DF1 bertambah berdasarkan keamatan jalur yang terhasil di atas membran dari pemedapan Western. Kesimpulannya, hasil-hasil dalam kajian ini menunjukkan kebolehan vektor diinkorporasi IRES yang dibina dari struktur plasmid laktokokkal mentranslasi kedua-dua protein yang diklon secara serentak di dalam eukariot lantas menjadikan ia boleh digunakan untuk pengekpresian gen antigen atau terapeutik untuk penghantaran vaksin dan protein bermanfaat ke dalam eukariot.

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I certify that a Thesis Examination Committee has met on 15 February 2016 to conduct the final examination of Nur Elina binti Abdul Mutalib on her thesis entitled "Development of IRES-Incorporated Lactococcal Bicistronic Vector for Gene Delivery into Eukaryotic Cells" 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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This is to confirm that:

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%	percent/percentage
µg	Microgram
BLAST	Basic local alignment search tool
bp	base pair
$CaCl_2$	Calcium chloride
cDNA	Complimentary DNA
CEF	Chicken embryo fibroblast
CO_2	Carbon dioxide
DAB	3,3'-diaminobenzidine
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	ethylene diamine tetraacetic acid
FBS	Fetal bovine serum
GFP	Green fluorescent protein
h	Hour
HRP	Horseradish peroxidase
IBDV	Infectious bursal disease virus
IRES	Internal ribosome entry site
Kb	Kilo base pair
kDa	Kilo Dalton
L	Liter
LB	Luria Bertani
LAB	Lactic acid bacteria
M	Molarity
$MgCl_2$	Magnesium chloride
mins	Minutes
ml	Milliliter
mM	miliMolar
mRNA	Messenger RNA

ng	nanogram
^{o}C	degrees centigrade
OD_{600}	Absorbance at 600 nm
ORF	Open reading frame
PBS	Phosphate buffered saline
P_{cmv}	Cytomegalovirus promoter
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PVDF	polyvinydene difluoride
rcf/ g	Relative centrifugal force
RE	Restriction enzyme
RNA	Ribonucleic acid
RRL	Rabbit reticulocyte lysate
RT	Room temperature
RT PCR	Reverse transcriptase PCR
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	Seconds
TAE	Tris-acetate EDTA
TE	Tris-EDTA
TEMED	tetramethyl-ethylene diamine
TGS	tris-glycine-SDS
UV	Ultraviolet
v/v	volume per volume
w/v	weight per volume
µl	Microliter

CHAPTER 1

INTRODUCTION

Internal ribosome entry sites (IRES) are RNA elements that direct internal initiation of translation (Kafasla *et al.*, 2009). It was first discovered in the RNA genome of picornaviruses, where the 5' untranslated region (UTR) from these particular viruses were able to mediate translation without the presence of methylated capped 5' mRNA (Jang et al., 1988). Conventional initiation of translation requires the methylation of the 5' end of mRNA known as capped mRNA which will interact with the complexes of eukaryotic initiation factor, eIF4F. This particular eIF is a protein complex consisting of several other eIFs, particularly eIF4G which act as one of the limiting factors for the binding between ribosomal subunit-eIFs to capped end of mRNA (Kozak, 1992).

Another method to initiate mRNA translation is IRES-mediated translation where the ribosomes bind to an uncapped internal mRNA. This particular process of initiation of translation differs slightly or greatly depending on the types of IRESs used, where some eIFs were not required for the translation initiation and the ribosomal subunit will bind to an internal site of the mRNA, instead of binding at 5' end of the mRNA structure. First discovered in RNA viruses, particularly at the untranslated region (UTR) of the viral genome, IRESs act as cis-acting element that attract ribosomes to bind at internal mRNA site enabling translation of any ORF downstream of the IRES element.

In a recent development, IRESs and its derivatives are now used as the platform for the construction of bicistronic or polycistronic expression vector to drive multiple gene expression from a single mRNA based on both cap-dependent and IRES-mediated translation. The basic of utilizing the IRES element in promoting cap-independent translation is the composition of the genes. The placement of the IRES element was between dicistronic regions. The first coding region was followed by another intercistronic region. Translation of the upstream cistron relies on cap-dependent translation, whereas translation of the other cistron relies on the presence of an internal ribosome entry site (Attal *et al.*, 1999; Hennecke *et al.*, 2001).

Most delivery methods of DNA vectors into eukaryotic cells were performed by co-transfection of 2 different plasmids; one carrying the gene of interest and the other gene encoding for a reporter protein (Sun *et al.*, 2005). Expression of reporter gene will mark the possible successful transfection of the plasmid carrying gene of interest as well, but the success rate is inconsistent and mostly gave false positive transfection results. Incorporation of IRES in eukaryotic expression vectors will eliminate the uncertainty of the transfection analysis where both genes of interest were cloned under different translation regulatory element/ or placed downstream of different promoters in a single vector.

Lactic acid bacteria (LAB) are groups of bacteria normally associated with food fermentation. It is safely consumed by humans and animals alike, thus making it

potential candidates for developing oral vectors, replacing the use of attenuated pathogens and advancing the development of mucosal vaccine (Bermúdez-Humarán *et al.*, 2011). The model organism for LAB is *Lactococcus lactis* and its vectors have been widely used in various applications ranging from developing new types of cloning and expression vectors, production of beneficial proteins and vaccines and utilizing *L. lactis* as mediator for delivery proteins and genetic materials to eukaryotes (Bahey-El-Din, 2012).

Bacteria-mediated plasmid transfer using *L. lactis* or LAB to eukaryotic cells has also been explored recently. Previously, this particular transfer was performed using attenuated pathogenic bacteria due to their ability to invade or internalized into eukaryotic cells. The mechanism of the delivery is still unconfirmed, but all the reports agreed with the same findings; *L. lactis* can mediate the delivery of plasmid DNA from its cells to the eukaryotic cells *in vivo* and *in vitro*, although it is non-pathogenic and non-invasive in nature (Guimarães *et al.*, 2006a; Innocentin *et al.*, 2009; Tao *et al.*, 2011).

Over the years, the development of eukaryotic expression plasmids from lactococcal plasmid backbone were reported (Glenting et al., 2002; Gram et al., 2007; Guimarães et al., 2009, 2006a; Tao et al., 2011). The vectors developed from these previous studies were monocistronic vectors primarily tested for its ability to mediate cap-dependent translation of a reporter gene. However, no reports are available on the development of bicistronic eukaryotic vecotrs from a lactococcal vector DNA backbone; either vector containing dual promoters or incorporated with IRES.

Therefore, the objectives of this study were:

1. To construct a bicistronic vector consisting of an eukaryotic expression cassette consisting of the cytomegalovirus promoter (P_{cmv}), terminator transcription signal polyA and internal ribosome entry site (IRES) and 2 open reading frames (ORFs) encoding the VP2 gene of infectious bursal disease virus (IBDV) and green fluorescent protein (GFP) into a lactococcal DNA vector, pNZ8048.

2. To analyse the ability of the constructed bicistronic vector to perform dual gene expression in a cell-free lysate.

3. To transfect a chicken cell line the with the bicistronic vector using jETPRIME transfection reagent.

4. To transfect DF1 cells with the bicistronic vector via bactofection using *L. lactis* NZ9000 cells as the bacteria carrier.

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