UNIVERSITI PUTRA MALAYSIA

CLONING AND EXPRESSION OF NUCLEOCAPSID PROTEIN OF NEWCASTLE DISEASE VIRUS FUSED WITH VP5 GENE OF INFECTIOUS BURSAL DISEASE VIRUS

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FBSB 2007 16

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2007

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By

SHAHERNY ZAID

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

June 2007

This thesis is especially dedicated to:

My Mom and family, who are infinitely very precious to me.

And

to my beloved, Zulkefley Othman and my lovely daughter, Nurqistina Batrisya who have colored my life with joy and happiness.

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Chairman: Datin Paduka Professor Khatijah Mohd. Yusoff, PhD

Faculty: Biotechnology and Biomolecular Sciences

Infectious Bursal Disease Virus (IBDV) is a constant threat to the poultry industry worldwide. This virus encodes a non-structural protein VP5 (17 kDa) that is capable of inducing apoptosis and may play an important role in pathogenesis of IBD. The nucleoprotein (NP) of Newcastle Disease Virus (NDV) has been shown possible to be use as a general carrier. In this study, the C-terminal end of NP full-length (pTrcHis2) were fused to the full-length of VP5 of IBDV to study the effect of these construct in cell culture and the possibility of this recombinant to induce apoptosis. The clones were then expressed and solubility test were done to determine the level of solubility of the recombinant proteins by Western blot. The DNA insert of NPfl-VP5 and VP5 were further subcloned into pCDNA3.1/V5 TOPO TA vector. The pcDNA3.1-NPfl-VP5 and pcDNA3.1-VP5 constructs were tested for transient expression in Chinese hamster ovary (CHO) cells. DNA laddering analysis and Acridine orange propidium iodide (AOPI) was also performed in order to check for any changes due to apoptosis. Expression of the NPfl-VP5 protein in *E. coli* was not detected by SDS-PAGE but a band at the expected size (~

70-80 kDa) was detected using Western. Only 5% of this protein was soluble. The PCR products of NPfl-VP5 (1.9 kb) and VP5 (447 bp) were subcloned into pcDNA3.1 vector. The CHO cells that were used in *in vitro* expression were successfully transfected with the plasmid contain the inserts. After 48 hours post-transfection, the cells that were transfected with pcDNA3.1-NPfl-VP5 and pcDNA3.1-VP5 plasmids exhibited apoptosis. In contrast, no apoptosis was observed in the cells transfected with pcDNA3.1 vector only. To confirm that apoptosis had occurred, at 48 hours post-transfection, the cellular DNA was extracted and analyzed by 2% agarose gel electrophoresis. A laddering effect indicative of nucleosomal fragmentation, was detected in the DNA samples obtained from the cells transfected with pcDNA3.1-NPflVP5 or with pcDNA3.1-VP5 but not with the samples from cells transfected with pcDNA3.1 vector only. The expression of NPfl-VP5 and VP5 proteins in transfected CHO cells were detected by Western blot analysis using anti-V5, anti-NDV, anti-IBDV and anti-VP5 antibodies. Further investigation using AOPI methods also confirm the apoptosis effects. Statistical analysis showed that the percentage of apoptosis cells was significantly higher when transfected with pcDNA3.1-NPflVP5 compare with pcDNA3.1-VP5. As a conclusion, the recombinant protein was successfully constructed and NP can be used as a carrier, and the fusion of NP to VP5 will induce more apoptosis. However, it just slightly increases its solubility. Thus, the results showed that recombinant protein NPfl-VP5 and also VP5 alone were able to induce apoptosis in cell culture.

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

PENGKLONAN DAN PENGEKSPRESAN GABUNGAN PROTIN NUKLEOCAPSID VIRUS PENYAKIT NEWCASTLE DENGAN GEN VP5 VIRUS PENYAKIT BURSA BERJANGKIT

Oleh

SHAHERNY ZAID

June 2007

Pengerusi: Datin Paduka Profesor Khatijah Mohd. Yusoff, PhD

Fakulti: Biotechnologi dan Sains Biomolekul

Virus penyakit bursa berjangkit (IBDV) merupakan ancaman yang berterusan kepada industri ternakan ayam seluruh dunia. Virus ini mempunyai protein yang tidak berstruktur VP5 (17 kDa) yang mampu untuk meransang apoptosis dan mungkin memainkan peranan penting didalam proses patogenesis IBD. Nukleoprotein (NP) Virus Penyakit Newcastle (NDV) telah dikatakan berkemungkinan berguna sebagai pembawa umum. Oleh itu, didalam kajian ini, hujung terminal-C NP (pTrcHis2) telah digabungkan dengan VP5 penuh untuk mengkaji kesan penggabungan ini didalam kultur sel dan kemungkinan gabungan ini untuk meransang apoptosis. Klon-klon ini telah diekspreskan dan ujian kelarutan telah dilakukan untuk menentukan tahap kelarutan protein rekombinan dengan Western blot. Seterusnya NPfl-VP5 dan VP5 telah disubklon ke dalam vector pCDNA3.1/V5 TOPO TA yang kemudiannya telah di uji untuk pengekspresan ‘transient’ didalam sel Chinese hamster ovary (CHO). Analisis ‘DNA Laddering’ dan ‘Acridine orange propidium iodide’ (AOPI) juga telah dijalankan untuk meneliti sebarang perubahan yang disebabkan oleh apoptosis. Pengekspresan

protein NPfl-VP5 didalam *E. coli* tidak dapat dikesan menggunakan SDS-PAGE tetapi jalur saiz yang dijangka (70 ke 80 kDa) telah dikesan dengan menggunakan Western blot. Hanya 5% daripada protein larut. Produk PCR NPfl-VP5 (1.9 kb) dan VP5 (447 kb) telah disubklon ke dalam vector pcDNA3.1. Selepas 48 jam setelah sel CHO ditransfeksi secara *in-vitro*, sel-sel yang ditransfekkan dengan plasmid pcDNA3.1-NPfl-VP5 dan pcDNA3.1-VP5 menunjukkan apoptosis. Sebaliknya, tiada apoptosis dilihat didalam sel-sel yang ditransfekkan dengan kawalan. Untuk memastikan yang apoptosis telah berlaku pada 48 jam selepas transfeksi, DNA selular telah diekstrak dan dianalisa menggunakan 2% gel agarose elektroforesis. Kesan "bertangga" menandakan pemecahan nukleosomal telah dikesan didalam sampel DNA yang didapati dari sel-sel yang ditransfekkan dengan pcDNA3.1-NPflVP5 atau dengan pcDNA3.1-VP5 tetapi tidak dengan sampel dari sel-sel yang ditransfekkan dengan kawalan. Pengekspresan protein NPfl-VP5 dan VP5 didalam sel-sel CHO yang ditransfekkan telah dikesan menggunakan analisa Western blot menggunakan antibodi anti-V5,anti-NDV,anti-IBDV dan anti-VP5. Kajian AOPI juga mengesahkan kesan apoptosis. Analisis statistik telah menunjukkan bahawa peratus sel apoptosis secara signifikan adalah lebih tinggi bila ditransfekkan dengan pcDNA3.1-NPfl-VP5 berbanding pcDNA3.1-VP5. Sebagai kesimpulan, protein gabungan telah berjaya dibentuk dan diekspreskan, dan gabungan NP dan VP5 akan lebih merangsang apoptosis. NP boleh digunakan sebagai pembawa walaupun hanya meningkatkan sedikit kelarutannya. Keputusan juga menunjukkan yang protein recombinan NPfl-VP5 dan juga VP5 mampu untuk meransang apoptosis didalam kultur sel.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank all people who have helped me throughout the completion of my Master's thesis. Without them, this project would not have been possible.

First of all, I owe my indebtedness to my wonderful supervisor, Prof. Datin Dr. Khatijah Mohd. Yusoff for introducing me to this field and also for all the personal attention, excellent advise, guidance, encouragement and support throughout my research. I also would like to thank my co-supervisors, Assoc. Prof. Dr. Abdul Rahman Omar and Dr. Muhajir Hamid for their invaluable comments and suggestions. I also would like to extend my sincere thanks to my employer, Universiti Pendidikan Sultan Idris (UPSI) and JPA for their sponsorship for my Master's programme.

I also would like to acknowledge with sincere gratitude to Dr. Majid Eshagi for guiding me in tissue culture work. Special thanks to Wan Key Fei for providing me the cells for my research and also Prof. Manaf Ali and his students in guiding me in the usage of the fluorescence microscopy.

I am grateful to my laboratory mates, especially to Fida, Sue, TC, Andrew, Wawa, Taz, Kak Tan, Lalita, Kah Fai, Sing King, Firoozeh, Eddie, Mok, Max, Yeng Peng, Swee Tin and Chew Ling for assisting me in various ways in my laboratory work and for giving me motivation. Appreciation also goes to Nazrien and Tajul for guiding me in AOPI.

I extend my deepest and warmest thank to my beloved, Zul and my family for being so helpful, understanding and for having patience in me. Not forgetting, thanks to all my friends who were always being there for me during the hard times of my research.

Finally, above of all, I thank God for His blessing on me….

I certify that an Examination Committee has met on 27th June 2007 to conduct the final examination of Shaherny binti Zaid on her Master of Science thesis entitled "Cloning and Expression of Nucleocapsid Protein (NP) of Newcastle Disease Virus Fused With VP5 Gene of Infectious Bursal Disease Virus" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Science.

Members of the Examination Committee were as follows:

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Faculty of Biotechnology and Biomolecular Sciences
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Date:

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

Khatijah Mohd Yusoff, PhD
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Date: 22 January 2008

DECLARATION

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

_____

SHAHERNY ZAID

Date:

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LIST OF ABBREVIATIONS

amp	ampicillin
AOPI	Acridine orange propidium iodide
ATP	Adenosine triphosphate
β-Gal	β-Galactosidase
bp	base pair
C	Carboxy
cDNA	copy DNA
CMV	Cytomegalovirus
CO_2	Corbon dioxide
DMSO	Dimetyl sulfoxide
DNA	Deoxyribonucleic Acid
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
EDTA	Ethylene diamenetetraacetate
EM	Electron microscopy
FBS	Foetal bovine serum
HCl	Hidrocloric acid
IBD	Infectious Bursal Disease
IBDV	Infectious Bursal Disease Virus
kb	kilobase
kDa	kilo Dalton
Mab	Monoclonal antibody
mRNA	messenger RNA
NaCl	Sodium chloride

NDV	Newcastle Disease Virus
nt	nucleotide
OD	Optical density
OIE	Office International des Epizooties
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
p.i.	post-infection
RdRp	RNA-dependent RNA polymerase
RNA	Riboucleic acid
RNAse	Ribonuclease
rpm	Rotation per minute
SDS-PAGE	Sodium dodecyl sulphate - Polyacrylamide Gel Electrophoresis
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
UPM	Universiti Putra Malaysia
VLP	Virus-like Particle
VP	Viral protein
VPg	genome-linked viral protein
vvIBDV	very virulent IBDV

CHAPTER 1

INTRODUCTION

Infectious bursal disease virus (IBDV) is a major important pathogen to the poultry industry worldwide, causing a highly contagious immunosuppressive disease in young chickens by destroying the lymphoid cells in the bursa of Fabricius (BF) (Mundt *et al*.,1995). In addition, the immunosuppression increases the susceptibility of these chickens to other infections and interferes with vaccination against other diseases (Saif,1991).

IBDV is a small, icosahedral and non-enveloped virus which belongs to the genus *Avibirnavirus* of the family *Birnaviridae* (Murphy *et al*., 1995). There are two serotypes of IBDV; serotype 1 strains are pathogenic to chickens whereas serotype 2 strains are not (Ismail *et al.,* 1988). The virus contains a bisegmented double-stranded RNA genome, namely segments A and B. The larger Segment A encodes three structural virion proteins, VP2, VP3 and VP4, and a nonstructural protein, VP5 (Mundt *et al*., 1997). The smaller segment B, encodes VP1, the viral RNA polymerase (Muller & Nitschke, 1987). VP2 has been identified as the host protective antigen as it contains the antigenic regions responsible for the induction of neutralizing antibodies (Fahey *et al*., 1989). VP3 is considered to be a group specific antigen as it is only recognized by non-neutralizing antibodies, some of which cross-react with serotypes 1 and 2 (Oppling *et al*., 1991). VP4 is a viral protease involved in the processing of the polyprotein (Azad *et al*., 1987). It has been

demonstrated that VP5 is neither required for the viral replication *in vitro* (Mundt *et al*., 1997) nor *in vivo* but plays an important role in pathogenesis as proven by the generation of a VP5-defective virus which had lost ability to cause bursal lesions (Yao *et al*., 1998).

The use of a reverse genetics system has allowed the generation of a VP5 knockout mutant. Work with this VP5 deletion mutant has shown that VP5 is dispensable for virus replication *in vitro* (Mundt *et al.,* 1997). Interestingly, this mutant showed a highly attenuated phenotype which did not cause bursal lesions after experimental infection of susceptible chickens, demonstrating that VP5 plays a key role in IBDV pathogenesis and dissemination (Yao *et al.,*1998). Consequently, VP5 has attracted attention as a potential target in the development of strategies to control IBDV. The study by Yao et al. (1998) indicated that the absence of VP5 protein expression in the mutant virus will not affect the humoral immune response to IBDV in the natural host. Therefore, it may be feasible to develop a novel, live-attenuated vaccines for IBDV that are non-pathogenic to chickens. Although several improvements in the control of the diseases by vaccination have been obtained, the results remain unsatisfactory. Despite strong interest in VP5, the Birnavirus life cycle has not been characterized in detail. Very little is known on the virus entry, genome transcription and replication, morphogenesis, and release of the viral progeny.

Studies have shown that immunosuppresssion induced by IBDV is caused, at least in part, by apoptosis or programmed cell death (Tanimura & Sharma,

1998). Recently, it has been shown that an IBDV infection induces apoptosis in chicken embryo and tissue culture cells (Yao and Vakharia, 2001). VP5 alone is capable of inducing apoptosis in cell culture. Transfection of chicken B-lymphocyte cell line (RP9) and chicken embryo fibroblast cells with a plasmid DNA containing the VP5 gene under the control of the intermediate-early promoter enhancer region of human cytomegalovirus, induced apoptosis in both cell lines. Apoptotic changes such as chromatin condensation, DNA fragmentation, and the appearance of apoptotic nuclear bodies were observed in cell cultures 48-h post-transfection (Yao and Vakharia, 2001). In addition, induction of VP5 expression also resulted in the alteration of cell morphology, the disruption of the plasma membrane, and a drastic reduction of cell viability. These observations indicate that the VP5 is highly cytotoxic and is involved in the release of the viral progeny from cells, an important role in viral pathogenesis. (Lombardo *et al*., 2000; Yao and Vakharia., 2001).

The nucleoprotein (NP) of Newcastle disease virus (NDV) has recently been expressed in *Escherichia coli* where it assembles into ring-like particles. It has been suggested that the NP has the potential to be developed as a carrier for chimeric antigens (Kho *et al*, 2001b). In addition, it can be expressed in abundance as a highly soluble protein. This protein is stable and has its C-terminus exposed making it ideal for carrying any antigen or multiple antigens (Rabu *et al*, 2002). Therefore, it would be interesting to know whether the fusion of NP with VP5 might increase the efficacy of the latter in inducing apoptosis compared to VP5 alone, since fusion of NP with

HN and F proteins of NDV induced significant levels of antibodies against NDV in chickens (Rabu *et al*., 2002). With the use of NP as a carrier, NP-VP5 might be expressed in soluble and stable form due to the ability of NP, which can act as a carrier for other peptide (Rabu *et al*., 2001).

Despite a number of studies on IBDV, very little is known about the expression of the VP5 gene of IBDV or on a recombinant VP5. In this study, the full length of VP5 were fused to the C-terminal of NP to study the role and effect of chimera NP-VP5 and a possibility of this chimera could induce apoptosis. Hence, the purpose of fusing NP of NDV with VP5 of IBDV in this study was to increase the efficacy of VP5 in inducing apoptosis in normal mammalian cells.

Therefore the objectives of this study are:

1. to construct recombinant DNA molecules carrying NP of NDV and VP5 gene of IBDV;
2. to express the chimeric protein in a mammalian expression system; and to study its effect on apoptosis *in vitro.*

CHAPTER 2

LITERATURE REVIEW

2.1 IBDV and the disease

Infectious bursal disease (IBD) is a highly contagious, immunosuppressive disease of young chickens caused by the infectious bursal disease virus (IBDV). The disease is characterized by destruction of the lymphoid cells in the bursa of Fabricius (BF), which leads to severe B-cell suppression (Becht, 1980; Kibenge *et al*., 1988) resulting in susceptibility to other infections and interferences with vaccination programmes (McFerran *et al*., 1980; Snyder *et al*., 1988). The infection depends on the dose and virulence of the strain, the age and breed of animals, and the presence or absence of passive immunity (Tacken *et al*., 2000). Nevertheless, the disease has no direct impact on public health as there is no evidence of transmission of IBDV to other non-avian species including human.

2.2 History and epidemiology

The disease was discovered in 1962 in Gumboro, Delaware, USA (Cosgrove, 1962) but had been present since 1957 (Cover, 1960). Later outbreaks were subsequently referred to as 'Gumboro Disease' after the geographic location. Originally the condition was named 'nephritis-nephrosis syndrome of chicken' because of prominent kidney lesions. After it became evident that an