
**UNIVERSITI PUTRA MALAYSIA**

# *CLONING AND EXPRESSION OF THE SPIKE PROTEIN OF NEPHROPATHOGENIC INFECTIOUS BRONCHITIS VIRUS STRAIN MH5365/95*

**YAP MAY LING**

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# CLONING AND EXPRESSION OF THE SPIKE PROTEIN OF NEPHROPATHOGENIC INFECTIOUS BRONCHITIS VIRUS STRAIN MH5365/95

**By**

**YAP MAY LING**



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

**September 2004**

DEDICATED TO……..

My Late Father,

YAP KAM SANG

My Mother,

CHAN HA MOOI

My Brothers and Sister,

KOON HOI,

KOK HOU,

KOK WAI,

MEI YUEN.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

**CLONING AND EXPRESSION OF THE SPIKE PROTEIN OF NEPHROPATHOGENIC INFECTIOUS BRONCHITIS VIRUS STRAIN MH5365/95**

By

**YAP MAY LING**

**July 2004**

**Chairman: Associate Professor Siti Suri Arshad, Ph.D.**

**Faculty: Veterinary Medicine**

Infectious bronchitis is a highly contagious respiratory and kidney disease of poultry. The etiological agent, the prototype coronavirus, infectious bronchitis virus (IBV) is a member of the family *Coronaviridae*. The objective of this research is to isolate S1 protein from nephropathogenic IBV strain MH5365/95. The spike (S1) gene encodes for the S1 surface glycoprotein which is involved in virus attachment and infectivity. In addition, the S1 possesses the main immunological determinants essential for immune response and host protection. Production and isolation of the S1 protein from the rest of viral immunogens is necessary for further subunit vaccine development and protein structure-functional studies. To achieve these, therefore, the S1 gene of the IBV strain MH5365/95 was cloned into *Escherichia coli* expression system and also into the Baculovirus Expression Vector System (BEVS). This is the first study ever conducted in Malaysia in which the viral immunogen of a local IBV strain was cloned and expressed as a recombinant protein in heterologous cell system. The 1.75 kb S1 gene

was amplified from the viral genomic RNA by RT-PCR method. It was cloned into the *E. coli* expression vector, pGEX-2T, and the baculovirus transfer vector, pAcG-2T. The recombinant clones were verified by restriction enzyme analysis, PCR and partial DNA sequencing. The recombinant S1 was expressed as glutathione S-transferase (GST) fusion protein in both the expression systems. In *E. coli* cells, the GST-S1 fusion protein was expressed at a relatively low level despite the optimization studies done. In Western blot analysis using an anti-GST polyclonal antibody, the *E. coli*-derived fusion protein was identified as a protein band having a molecular weight of approximately 90 kDa. Upon co-transfection of the recombinant baculovirus transfer vector with BaculoGold® linearized baculovirus genomic DNA in *Sf*9 insect cells, a viable recombinant baculovirus AcNPV (recS1-AcNPV) was verified by PCR and purified through plaque assay. The recS1-AcNPV recombinant baculovirus failed to produce any occlusion bodies in *Sf*9 cells consequent upon the replacement of baculoviral polyhedrin gene by S1 gene. In western blot analysis, the *Sf*9-derived GST-S1 fusion protein was identified as a protein band with molecular weight of approximately 105 kDa, reacting with anti-GST polyclonal antibody. The size of the S1 moiety was estimated to be approximately 79 kDa. This result showed that level of glycosylation in the *Sf*9-derived S1 protein was not equal to that of the authentic S1 glycoprotein. Moreover, the same protein was unable to react with the polyclonal antiserum raised against IBV MH5365/95. Thus, further analysis on the glycosylation pattern, conformation and antigenicity of the S1 protein is necessary.

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

**PENGKLONAN DAN PENGEKSPRESAN PROTEIN PEPAKU VIRUS BRONKITIS BERJANGKIT STRAIN MH5365/95 NEFROPATOGENIK**

Oleh

**YAP MAY LING**

**Julai 2004**

**Pengerusi: Profesor Madya Siti Suri Arshad, Ph.D,**

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Bronkitis berjangkit merupakan penyakit pernafasan dan penyakit ginjal paling menular dalam ayam itik. Agen etiologinya, koronavirus prototip, iaitu virus bronkitis berjangkit (IBV) merupakan ahli famili *Coronaviridae*. Objektif penyelidikan ini adalah untuk mengasingkan protein S1 daripada virus bronkitis berjangkit strain MH5365/95 nefropatogenik. Gen pepaku (S1) mengekod untuk glikoprotein permukaan S1 yang terlibat dalam pelekapan dan kejangkitan virus. Sambil itu, ia juga mempunyai penentu imunologi utama untuk gerak balas imun dan perlindungan perumah. Penghasilan dan pemencilan protein S1 daripada imunogen virus lain adalah perlu untuk perkembangan vaksin subunit dan kajian struktur-fungsi protein seterusnya. Untuk mencapai matlamat ini, maka gen S1 bagi strain IBV MH5365/95 telah diklonkan ke dalam sistem pengekspresan *Escherichia coli* dan juga ke dalam Sistem Vektor Pengekspresan Bakulovirus (BEVS). Ini merupakan pengajian terulung yang dijalankan di Malaysia di mana imunogen virus strain IBV tempatan diklon dan diekspreskan sebagai protein rekombinan di dalam sistem sel heterologos. Gen S1 bersaiz 1.75 kb ini telah

diamplifikasi daripada RNA genom virus melalui kaedah RT-PCR. Gen ini diklonkan ke dalam vektor pengekspresan *E. coli*, pGEX-2T, dan vektor pemindahan bakulovirus, pAcG-2T. Klon rekombinan ini telah disah betul melalui analisis enzim penyekat, PCR dan penjujukan DNA separa. S1 rekombinan telah diekspres sebagai protein lakuran glutation S-transferase (GST) dalam kedua-dua sistem pengekspresan tersebut. Dalam sel *E. coli*, protein lakuran GST-S1 telah diekspres pada aras agak rendah walaupun kajian pengoptimuman telah dijalankan. Dalam analisis pemblotan Western mengguna antibodi poliklon anti-GST, protein lakuran terbitan *E. coli* ini telah dikenal pasti sebagai jalur protein yang berat molekulnya lebih kurang 90 kDa. Selepas ko-pentransjangkitan vektor pemindahan bakulovirus rekombinan dengan DNA genom bakulovirus linear BaculoGold® ke dalam sel serangga *Sf9*, satu bakulovirus rekombinan AcNPV (recS1-AcNPV) telah disah betul melalui PCR dan ditulenkan melalui assai plak. Bakulovirus rekombinan recS1-AcNPV gagal untuk menghasilkan jasad oklusi dalam sel *Sf9* kerana telah berlaku penggantian gen polihedrin bakulovirus dengan gen S1. Dalam analisis pemblotan Western, protein lakuran GST-S1 terbitan *Sf9* telah dikenal pasti sebagai jalur protein berat molekul lebih kurang 105 kDa yang bertindak balas dengan antibodi poliklon anti-GST. Saiz moieti S1 ini dianggarkan lebih kurang 79 kDa. Hasil kajian ini menunjukkan yang aras pengglikosilatan dalam protein S1 terbitan *Sf9* tidak sama dengan glikoprotein S1 asli. Kajian menunjukkan bahawa protein yang sama tidak mampu untuk bertindak balas dengan antiserum poliklon yang dihasilkan terhadap IBV MH5365/95. Dengan demikian, analisis lanjutan terhadap pola pengglikosilatan, konformasi dan keantigenan protein S1 adalah perlu.

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I certify that an Examination Committee met on **23th July 2004** to conduct the final examination of **Yap May Ling** on her **Master of Science** thesis entitled "Cloning and Expression of the Spike Protein of Nephropathogenic Infectious Bronchitis Virus Strain MH5365/95" 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 been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

**YAP MAY LING**

Date:

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| | |
|---|---|
| ~ | approximately |
| AcNPV | *Autographa californica* nuclear polyhedrosis virus |
| AcMNPV | *Autographa californica* multiple nucleocapsid per virus |
| AF | allantoic fluid |
| AMV | avian myeloblastosis virus |
| ATP | adenosine triphosphate |
| BCIP | 5-bromo-4-chloro-3-indolyl-phosphate |
| BEVS | Baculovirus Expression Vector System |
| bp | base pair |
| BSA | bovine serum albumin |
| BV | budded virus |
| $^{o}C$ | degrees centrigrade |
| CDNB | 1-chloro-2,4-dinitrobenzene |
| $cm^2$ | centimeter square |
| CPE | cytopathic effect |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| dpi | day-post-infection |
| dNTP | deoxyribonucleotide triphosphate |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| ECV | extracellular virus |
| FBS | fetal bovine serum |
| GST | glutathione S-transferase |
| GV | granulosis virus |
| h | hour |
| HA | haemagglutination |
| HI | haemagglutination-inhibition |
| hpi | hour-post-infection |
| IB | infectious bronchitis |
| IBV | infectious bronchitis virus |
| IgG (H+L) | immunoglobulin G (high and low) |
| IPTG | isopropyl 1-thio-β-D-galactoside |
| kb | kilobase pair |
| kDa | kilodalton |
| LB | Luria Bertani |
| M | membrane |
| M | molar |
| µg | microgram |
| µl | microliter |
| µm | micrometer |
| mA | milliampere |
| MAb | monoclonal antibody |

| | |
|---|---|
| MCS | multiple cloning site |
| mg | milligram |
| min | minute |
| ml | milliliter |
| mm | millimeter |
| mM | millimolar |
| m.o.i. | multiplicity of infection |
| MOPS | 3-*N*-morpholino propanesulfonic acid |
| mRNA | messenger ribonucleic acid |
| MTA | methylamine tungstate |
| MW | molecular weight |
| N | nucleocapsid |
| NBT | nitro blue tetrazolium |
| nm | nanometer |
| NOV | nonoccluded virus |
| NPV | nuclear polyhedrosis virus |
| NTE | natrium chloride-tris-EDTA buffer |
| OB | occlusion body(ies) |
| OD | optical density |
| ORF | open reading frame |
| ori | origin of replication |
| OV | occluded virus |
| % | percentage |
| PAb | polyclonal antibody |
| PBS | phosphate buffer saline |
| PCR | polymerase chain reaction |
| PFU | plaque forming unit |
| *polh* | polyhedrin |
| PVDF | polyvinyl difluoride |
| RBS | ribosome binding site |
| RE | restriction enzyme |
| RNA | ribonucleic acid |
| rpm | round per minute |
| rS1-AcG | recombinant plasmid with S1 gene, derived from pAcG-2T vector |
| rS1-GEX | recombinant plasmid with S1 gene, derived from pGEX-2T vector |
| RT | room temperature |
| RT-PCR | reverse transcriptase-polymerase chain reaction |
| s | second |
| S | spike |
| S1 | spike subunit 1 |
| S2 | spike subunit 2 |
| SDGC | sucrose density gradient centrifugation |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| *Sf*9 | *Spodoptera frugiperda* cell line |
| TAE | tris-acetate-EDTA buffer |

| | |
|---|---|
| TE | tris-EDTA buffer |
| TEM | transmission electron microscopy |
| TEMED | *N*,*N*,*N'*,*N'*-tetramethylethylene diamine |
| U | unit |
| UV | ultraviolet |
| V | volt |
| VN | virus-neutralization |
| v/v | volume per volume |
| w/v | weight per volume |

# CHAPTER 1

# INTRODUCTION

## 1.1 Infectious Bronchitis

The poultry industry constitutes a significant sector in world agriculture. In United States, more than 8 billion birds are produced yearly with a value exceeding $20 billion (Sharma, 1999). In Malaysia, 644.56 thousand million tan of poultry meat and 6,534 million of chicken or duck eggs were consumed in year 2001 (Department of Veterinary Services Malaysia, 2002). The industry relies on intensive farming to supply meat and eggs at relatively low cost.

Infectious bronchitis virus (IBV), a member of family *Coronaviridae* and prototype of coronavirus, is the etiological agent of infectious bronchitis (IB), a highly contagious respiratory, kidney and urogenital tract disease in chickens. IB is prevalent in all countries with intensive poultry industries, with the incidence of infection approaching 100% in most locations (Ignjatovic and Sapats, 2000). It has a significant economic impact; in broilers, production losses are due to poor weight gains, condemnation at processing and mortality, whilst in laying birds, losses are due to suboptimal egg production and downgrading of eggs.

Effective vaccines live attenuated and inactivated oil adjuvanted against IB have been made available in Malaysia for many years (Heng *et al*., 1980; Aziz *et al*., 1996; Azri *et al*., 1996). However, problems are still encountered since there is recurrence of outbreaks in the vaccinated flocks, which are often accompanied by the isolation of more virulent isolates. The apparent continuous antigenic modifications of IBV that occurs in field situation often resulted in the generation of antigenically different serotypes as well as newly emerged variants, which are responsible for the 'vaccine breaks'. In 1995, a Malaysian nephropathogenic (kidney-ailment form) IBV strain causing high mortalities with nephrosis-nephritis syndrome among broiler chickens was isolated at Manjung, Perak (Aziz *et al*., 1996). Subsequent analysis of this isolate, designated as MH5365/95, showed that it is antigenically distinct from all known IBV serotypes including the vaccinal strains (Maizan, 2000). Therefore, development of more effective vaccines derived from this local IBV strain has become our main interest. This research project is a continuation study from a previous study carried by Maizan Mohamed (Maizan, 2000) in which the detection and molecular identification of IBV isolated in Malaysia were conducted.

Classically, detailed molecular analysis of proteins or other constituents of most organisms was rendered difficult or impossible by their scarcity and the consequent difficulty of their purification in large quantities. The development of techniques and methods for the separation and purification of biological macromolecules has been an important prerequisite to many of the advancement made in biotechnology since the 1950's.

Since *in vitro* manipulation of DNA through the use of polymerase chain reaction (PCR) was developed, the utility of recombinant expression systems for the production of useful proteins has been significantly expanded. Besides, recombinant DNA techniques may completely revolutionize the way vaccines are developed and used. They offer potentially purer, safer and of greater efficacy than many currently used vaccines. Determinants of important protective antigens from a variety of viral, bacterial and parasitic pathogens have been successfully cloned and expressed in novel hosts. Several host systems are available including phage, bacteria, yeasts, plants, filamentous fungi, and insect or mammalian cells grown in culture.

The prokaryotic *Escherichia coli* expression system is often the first choice when performing recombinant protein production studies due to its potentially high level of production and ease of propagation (Hooley and Burns, 1993; Prokop *et al*., 1991). Besides, there is a rich abundance of bacterial vector choices for this low-cost expression system. Many procedures for purification of recombinant proteins exist and this made advanced molecular analysis and further applications using the pure recombinant proteins possible.

After transcription and translation in the heterologous cell system, the amino acid or polypeptide chain produced may undergo a very wide range of post-translational modifications such as glycosylation and phosphorylation. These modifications varies with the type of cells, with cells from more complex organisms producing more complex modifications and can have major effects on the structure and function of the

proteins. Since it is important to obtain protein with properties similar to those authentic proteins as produced in original host cells, especially in vaccine formulation, it is often necessary to express the gene of interest originating in higher life forms such as in an eukaryotic system. Hence, beside the cheaper and more established prokaryotic *E. coli* expression system, the popular Baculovirus Expression Vector System (BEVS) was also included for recombinant protein expression in this study. BEVS was chosen due to its abundant expression of biologically active recombinant proteins that are soluble and antigenically, immunogenically and functionally similar to their authentic counterparts (O'Reilly *et al*., 1994).

## 1.2 Objectives of the Study

The production of pure recombinant S1 proteins of IBV strain MH5365/95 through the use of recombinant DNA technology would be advantageous under a variety of circumstances. It could be used to develop a subunit vaccine or for structural, functional and antigenic studies. Therefore the objective of this study is to express the S1 protein of Malaysian nephropathogenic IBV strain MH5365/95 by using two different recombinant protein expression systems, which are, the prokaryotic *E. coli* expression system as well as the eukaryotic BEVS.

This research, therefore, will comprise the following steps:

1) morphological study of the local nephropathogenic IBV strain MH5365/95,
2) isolation and amplification of the S1 gene from IBV genome using PCR method,

3) cloning of the S1 gene into *E. coli* expression vector and baculovirus transfer vector,
4) delivery of the recombinant vectors carrying the S1 gene into *E. coli* cells and *Spodoptera frugiperda* (*Sf*9) insect cells, and
5) expression of the recombinant S1 proteins in *E. coli* cells and *Sf*9 insect cells.

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