CLONING AND PRODUCTION OF FUSED PROTEIN CONSISTING OF VIRAL PROTEIN 2 FROM INFECTIOUS BURSAL DISEASE VIRUS AND HEMAGGLUTININ-NEURAMINIDASE FROM NEWCASTLE DISEASE VIRUS

BY

NOR AZLIN ALIA BINTI NOR MUHAMMAD

A thesis submitted in fulfilment of the requirement for the degree of Master of Health Sciences

Kulliyyah of Allied Health Sciences
International Islamic University Malaysia

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ABSTRACT

Malaysia is exploring opportunities in developing its poultry vaccination programme to produce better poultry vaccine to fight against the two most important diseases of poultry in Malaysia which is Newcastle disease (ND) and infectious bursal disease (IBD) which have been causing constant economic losses to the national livestock industry. Since the commercially available vaccines are consisting of less virulent virus strain that differs from the virulent outbreak strain, the safety and efficacy of the vaccines are becoming great concerns. Development of vaccines consisting of recombinant protein that contains epitopes which able to induce neutralizing antibodies are dominating in the strive for an ideal vaccine, being safe and cheap. Previous studies have shown that the viral surface proteins from Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV) contains epitopes which able to induce neutralizing antibodies against ND and IBD respectively. In this study, viral protein 2 (*VP2)* from IBDV and hemagglutinin-neuraminidase (*HN)* from NDV were isolated from IBDV and NDV of local isolates via RT-PCR and cloned into pCR2.1TOPO vector. Subsequently, two constructs of recombinant plasmid containing fused gene was constructed in which full length *HN* gene from NDV was fused to *VP2* of the infectious bursal disease virus (IBDV) while the other construct used partial *HN* gene. Production of the fused protein was attempted in *Pichia pastoris* using pPICZαC but was not successful. However, an intact fused protein of VP2-PHN constructed form VP2 and partial HN in pRSETB vector was successfully produced by the *Escherichia coli.* A protein band with expected molecular weight of 75 kDA was observed in SDS-PAGE and Western blot analysis upon detection with anti-Histidine monoclonal antibody. The VP2-PHN protein produced could be a potential candidate as recombinant subunit vaccine against both IBD and ND upon single immunization.

ملخص البحث

تعد ماليزيا من البلدان المهتمة بتطوير برنامج لقاحات الدواجن لإنتاج لقاح فعال وقادر على مكافحة أهم مرضين يصيبان الدواجن في ماليزيا وهما؛النيوكاسل وداء الغومبورو أو التهاب جراب فابريشيا الذين يتسببان بخسائر اقتصادية فادحةللإنتاج الماليزي الوطني في مجال تربية الدواجن. إن اللقاحات التجارية المتوفرة ضد هذه الأمراض مصنعة من فايروسات ذات سلالات أقل ضراوة وفتكا من تلك السلالات الفتاكة التي تصيب الدواجن، وقد أصبحت في مقدمة اهتمامات مكافحة هذه الأمراض أن تكون اللقاحات فعّالة وآمنة. إن فكرة تطوير اللقاحات التي تتكون من البروتين المؤتلف والمحتوية على حواتم)epitopes(قادرة علىإنتاج أجسام مضادة محايدةقد هيمنت على قطاع صناعة اللقحات كونها آمنة ورخيصة،وقد أثبتت دراسات سابقة أنّ البروتينات الفايروسية السطحية لداءالنيوكاسل وداء الغومبورو أو التهاب جراب فابريشيا تحتوي على حواتم)epitopes(قادرة على إنتاج أجسام مضادة للمرضين على التوالي.في الدراسة الحالية تمّ عزل البروتينات الفايروسية الثانية)VP2(من فايروس داء الغومبورو، وتم عزل)hemagglutinin-neuraminidase((HN) من فايروس داء نيوكاسل من سلالات محلية عبر تقنية)RT-PCR(واستنساخها في الناقل)pCR2.1TOPO(.بعد ذلك تم إنشاء بلازميدينِ مؤتلفينِ؛ الأول بلازميديحتوي على جين كامل من (HN) من فايروس نيوكاسل وقد تم دمجه مع)VP2(التابع لداءالغومبوروأو التهاب جراب فابريشيا، والثاني بلازميد مؤتلف يحتوي على جزء من جين (HN). وعلى الرغم من فشل عملية إنتاج بروتين منصهر في خميرة)Pichia pastoris(إلا أنّ النجاح كان حليفا في إنتاج)VP2-PHN(من)VP2(و)HN(الجزئي في الناقل)pRSETB(عن طريق بكتيريا)Escherichia coli(.إنّ حزمة من البروتين ذات وزن جزيئي مقدر بـ)57 kDA(قد تم ملاحظتها عن طريق)SDS-PAGE(، و) Western blot analysis(، عند التحري عنها مع الأجسام الأحادية المنشأ)monoclonal antibody(المضادة الهستادين. ويمكن القول إن بروتين)VP2-PHN(المنتج يمكن أن يكون مرشحا محتملا بوصفه لقاحا بروتينيا مؤتلفا ذا فعالية عالية ضد فايروسي لنيوكاسل والغومبورو.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Health Sciences.

……..……..
Raha Ahmad Raus
Supervisor

……..…….
Noriha Mat Amin
Co-Supervisor

……..…….
Norafiza Zainuddin
Co-Supervisor

……..…….
Farah Diba Abu Bakar
Co-Supervisor

I certify that I have read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Health Sciences.

……..……...
Munirah Binti Sha'ban
Internal Examiner

……..……...
Noraziah Mohamad Zin
External Examiner

This thesis was submitted to the Department of Biomedical Sciences and is accepted as a fulfilment of the requirement for the degree of Master of Health Sciences.

...……..……..
Ibrahim Adham Bin Taib
Head, Department of Biomedical Sciences

This thesis was submitted to the Kulliyyah of Allied Health Sciences and is accepted as a fulfilment of the requirement for the degree of Master of Health Sciences.

……..……..
Wan Azdie Bin Mohd. Abu Bakar
Dean, Kulliyyah of Allied Health Sciences

DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Nor Azlin Alia binti Nor Muhammad

Signature…… Date……..

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

CLONING AND PRODUCTION OF FUSED PROTEIN CONSISTING OF VIRAL PROTEIN 2 FROM INFECTIOUS BURSAL DISEASE VIRUS AND HEMAGGLUTININ-NEURAMINIDASE FROM NEWCASTLE DISEASE VIRUS

Affirmed by Nor Azlin Alia Binti Nor Muhammad.

……. ……

Signature Date

I would like to dedicate this research work to my mother (Zainun Hashim) and my father (Prof. Emeritus Dr. Nor Muhammad Mahadi) who are always there to love and support me throughout conducting this work,

I would also like to dedicate this work to my loving husband (Amirul Hakim Md Zubir), my beautiful daughter (Nor Anis Humaira Amirul Hakim), and my parent in-law (Hj. Md Zubir Ab Rahman & Hjh Nafsiah Hussin) who are very supportive and understanding,

Without all of you, I would not have made it. We made it!!

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LIST OF ABBREVIATIONS

APS	ammonium persulphate
BMGY	buffered glycerol-complex medium
BMMY	buffered methanol-complex medium
bp	base pair
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
°C	degree Celcius
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
edn.	edition
EDTA	ethylenediaminetetraacetic acid
et al.	(*et alia)*: and others
h	hour
IPTG	isopropyl-β-D-1-thiogalactopyranoside
kDa	kilo Dalton
LB	Luria-Bertani
mRNA	messenger ribonucleic acid
µFD	Faraday
µM	microMolar
µl	microlitre
µg/ml	microgram per millilitre
mg/ml	milligram per millilitre
mM	milliMolar
ml	millilitre
min	minute
M	Molar
MWCO	molecular weight cut-off
OD	Optical Density
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl fluoride
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolution per minute
RT-PCR	reverse transcriptase – polymerase chain reaction
s	second
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBST	Tris-buffered saline Tween 20
TEMED	tetramethylethylenediamine
UV	Ultraviolet
U/ml	Unit per millilitre
U/µl	Unit per microliter
V	voltage
YPD	yeast extract peptone dextrose

YPDS	yeast extract peptone dextrose sorbitol

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

The livestock industry in Malaysia continues to achieve strong growth. Poultry farming, which is the major sub-sector of livestock industry, contributes to the nation's agricultural output by producing meat and eggs from domesticated birds such as chickens, turkeys and ducks. Poultry farming contributes the largest percentage in the livestock industry with up to 53 %. It generates ex-farm value of RM10.6 billion for its product (Department of Veterinary Services [DVS], 2013). Currently, Malaysia is still exploring opportunities in developing its poultry vaccination programme to produce better poultry vaccine to fight against the two most important diseases of poultry in Malaysia which is Newcastle disease (ND) and infectious bursal disease (IBD) caused by Newcastle disease virus (NDV) and infectious bursal disease virus (IBDV) respectively, which have been causing constant economic losses to the national livestock industry (Hamidah, 2011).

Despite the continuous efforts in the vaccination programme, outbreaks of NDV and IBDV in vaccinated chickens of local farms still occurred (Lee et al., 2011; Kong et al., 2004; Ghazali et al., 2013). Outbreaks of IBD and ND also occurred worldwide in various countries as reported by previous studies. Several ND outbreaks have been reported in Netherlands (Dortmans et al., 2012), South Africa (Dortmans et al., 2014), and China (Zhang et al., 2014) while numerous IBD outbreaks also had occurred in countries such as China (Chen et al., 2012), Ethiopia (Jenberie et al., 2013), and India (Barathidasan et al., 2013). This calls for improvement in the current

vaccination programme such as the development of recombinant subunit vaccine.

Recombinant DNA technology and the production of recombinant proteins are the major breakthrough in molecular biology and have been applied in the development of recombinant subunit vaccine to find better alternatives for the production of efficient vaccines. Recombinant subunit vaccines are vaccines that are formulated with purified proteins that contain neutralizing epitopes capable of inducing antibodies against the virus (Nascimento & Leite, 2012). The basic principle for producing a recombinant subunit vaccine is to isolate and transfer the gene of interest into an expression host and followed by purification of the recombinant protein (or vaccine) produced by the host. The advantages of recombinant subunit vaccine are that the pathogen can be entirely excluded from the vaccine as well as eliminating the risks of reversion from non-virulent to virulent strains or incomplete inactivation of the virus (Liljeqvist & Stahl, 1999).

Many studies had been conducted to develop recombinant subunit vaccine by applying the recombinant DNA technology and expression of recombinant proteins. The first recombinant subunit vaccine was the Hepatitis B surface antigen vaccine produced in yeast (Valenzuela et al., 1982). A recent investigation on the development of recombinant subunit vaccine for the prevention of Hepatitis C virus (HCV) infections in human had been performed by producing the glycosylated enveloped proteins of E1 and E2 in *Pichia pastoris* expression system (Fazlalipour et al., 2014). In another study, a recombinant protein of hemagglutinin (HA) from influenza virus (H5N1) was produced in *Escherichia coli* and elicit neutralizing antibodies upon immunization that could be a potential recombinant subunit vaccine (Khurana et al., 2011).

1.2 STATEMENT OF RESEARCH PROBLEM

According to Malaysia's manufacturer of vaccines for poultry, currently the local poultry industry is administering two separate vaccines to protect the birds from NDV and IBDV. Live attenuated and killed vaccines have been manufactured for ND while only live attenuated vaccines are available for IBD (Malaysian Vaccines and Pharmaceuticals [MVP], 2012). To our knowledge, no bivalent subunit vaccines are commercially available for immunization of the poultry animals in Malaysia against these two major poultry diseases. The production of individual vaccines may elicit high production cost since the vaccines are produced from two separate processes.

Besides that, the use of viral vaccines imposes safety concern. Live attenuated vaccines are consisting of viruses belong to the less virulent strain that had been processed to reduce their pathogenicity in order not to cause disease in the host. Thus, one of the disadvantages of live attenuated vaccines is the risk of reversion into their original pathogenic forms. This was portrayed in the study of herpesvirus vaccine whereby the attenuated vaccine strains had resulted in virulent viruses that became the dominant strains responsible for widespread disease in Australian commercial poultry flocks (Lee et al., 2012). Moreover, the administration of viral vaccine into the chickens could cause the emergence of new strain due to recombination of the vaccine strain with the wild-type strain which also a potential safety concern (Kapczynski & King, 2005). Besides that, the preparation of live or killed vaccine requires proper containment and handling for the safety of personnel.

As mentioned before, outbreaks of ND and IBD still occurred worldwide in vaccinated poultry animals. This leads to the efficacy concern of the commercially available vaccines. Since the live attenuated vaccine is consisting of a less virulent virus strain, it might not be able to fully protect the chickens against the causative

agent strain which is highly pathogenic (Miller et al., 2013). As for killed vaccine, it poses efficacy concern because the preparation of the vaccine consists of harsh processes such as heating which might have damaged the coat or surface protein important for inducing the neutralizing antibodies (Meeusen et al., 2007). Therefore, this study attempts to produce recombinant subunit vaccine to overcome these disadvantages with addition that the subunit vaccine possesses two epitopes (VP2 from IBDV and hemagglutinin-neuraminidase, HN from NDV) to protect chickens against two different virus diseases.

1.3 IMPORTANCE OF STUDY

The production of recombinant fused protein consisting of VP2 from IBDV and HN from NDV would be a potential candidate as a recombinant subunit vaccine which would open more opportunities in developing a more effective vaccine to fight against IBD and ND in chickens upon single immunization. A vaccine developed by recombinant protein technology may overcome the economic and epidemiological safety problems associated with traditional vaccine preparations based on killed or attenuated virus.

Besides that, the recombinant fusion protein developed should be more effective as vaccine since both proteins fused are cloned from the same virus strain as the outbreak strain which is the highly pathogenic strain. It is also safer since it does not contain the virus genome or other attributes that can cause infections. Since the subunit vaccine will protect against both diseases simultaneously, there is no need to produce the vaccines for both diseases separately and thus, reduce the cost for vaccine production. Moreover, poultry farmers require purchasing only one vaccine instead of two vaccines to protect against ND and IBD. This investigation will definitely benefit

the poultry industry greatly.

1.4 OBJECTIVES OF STUDY

The general objective of this study was to produce a recombinant fused protein consisting of VP2 protein from IBDV and HN protein from NDV. The following are the specific objectives to support the general objective:

1. To isolate the *VP2* and *HN* genes from the RNA genome of IBDV and NDV, respectively.
2. To fuse *VP2* and *HN* genes in pRSETB expression vector
3. To subclone the fused *VP2-HN* gene into pPICZαC expression vector
4. To produce VP2-HN in *Pichia pastoris* and *Escherichia coli*

1.5 SCOPE OF RESEARCH WORK

This study involved the application of recombinant DNA technology in construction of a fused gene from two different viruses and the production of the resulting recombinant fused protein in two commonly used expression systems. To construct a fused viral gene, extraction of RNA genomes from IBDV and NDV were carried out using RNA extraction reagent. The IBDV isolate 3529/92 and the NDV isolate 7979 were provided by VRI of Malaysia. The isolation of *VP2* and *HN* genes from IBDV and NDV, respectively were performed by two major steps.

Firstly, *VP2* and *HN* genes were amplified from the RNA genome from each virus using specific primers via reverse-transcriptase polymerase chain reaction (RT-PCR). Subsequently, each gene was cloned into a cloning vector, pCR2.1TOPO before transformed into *E. coli* TOP10 strain. Prior to sequencing, the resultant recombinant plasmids were analysed via restriction enzymes by cutting the inserted

gene from vector.

According to the complete sequences of *VP2* and *HN* genes obtained, specific primers were designed to have restriction enzyme sites to fuse between the *VP2* gene and *HN* gene. Amplification of *VP2* gene from the previously constructed pCR2.1TOPO-VP2 was performed and cloned into pRSETB expression vector for *E. coli*. Following this, *HN* gene was fused to the resultant pRSETB-VP2 plasmids at the C-terminal of *VP2* gene using *KpnI* restriction site (which also act as a linker between *VP2* and *HN* genes). Two recombinant constructs were generated whereby one was consisting of full *HN* gene while the other was partial *HN* gene.

Previous studies have reported several successful attempts on the production of recombinant proteins isolated from viruses such as the production of viral protein 2 (VP2) protein from porcine parvovirus and envelope protein from Japanese encephalitis virus in *P. pastoris* methylotrophic yeast (Guo et al., 2014; Kwon et al., 2012) due to its advantages of post-translational modification and protein folding. To observe the production of VP2-HN protein in *P. pastoris* expression system, the resulting *VP2-HN* gene from recombinant pRSETB-VP2-HN plasmid was subcloned into pPICZαC *P. pastoris* expression vectors. Restriction enzyme analysis was carried out to confirm the presence of insert prior to the transformation into X-33 expression strain via electroporation. Under the control of *AOXI* promoter, expression of fused VP2-HN protein was attempted by induction with methanol. The pellet and supernatant of expressed transformants were subjected to SDS-PAGE and Western blot to analyse the presence of the fused protein.

Another alternative for expression of recombinant protein is using bacterial expression system. *E. coli* expression system remains the top choice due to its advantages such as inexpensive carbon source requirements, easy to scale up, and

rapid growth rates. Advance genetic strategies in recombinant protein expression have allowed efficient heterologous protein expression in *E. coli* with the presence of specifically designed expression host strain and vectors (Baneyx, 1999; Sorensen & Mortensen, 2005; Gopal & Kumar, 2013). Using *E. coli* BL21 (DE3) strain, the VP2-HN fused protein was expressed by pRSETB vector under the controlled of T7 promoter by induction with IPTG. The presence of expressed protein was confirmed via SDS-PAGE and Western blot through detection with anti-His monoclonal antibody.

CHAPTER 2

LITERATURE REVIEW

2.1 NEWCASTLE DISEASE

Newcastle disease (ND) is a highly contagious disease of birds caused by Newcastle disease virus (NDV). This disease is notorious for its devastations to the local commercial poultry industry which had caused significant economic losses. It is listed as one of the most notifiable terrestrial animal diseases by the World Organization for Animal Health (OIE) (2015). A number of cases of high mortality in broiler chickens have been reported in the local farms of Peninsular Malaysia which had various combinations of primary and booster vaccinations of ND with live lentogenic vaccine as well as inactivated ND vaccines (Lee et al., 2011). Some of the clinical signs (see Figure 2.1) are diarrhea, respiratory distress, central nervous system signs, and drop in egg production that influenced by the species infected, age and health of the host (Terregino & Capua, 2009).

Figure 2.1: Chickens infected with ND virus. The chickens infected exhibited clinical signs such as incoordination of muscular movement, distorted eggs, and paralysis (Terregino & Capua, 2009).