UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERIZATION AND EXPRESSION OF H5, N1 AND NS1 RECOMBINANT PROTEINS OF AVIAN INFLUENZA VIRUS SUBTYPE H5N1 IN Pichia pastoris

MUSTAPHA BALA ABUBAKAR

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By

MUSTAPHA BALA ABUBAKAR

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

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

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December 2011

Chairman: Professor Datin Paduka Aini Ideris, PhD

Faculty: Veterinary Medicine

Avian influenza (AI) subtype H5N1 is one of the major threats to the poultry industry with significant human health implication worldwide. It still remains the most feared poultry disease in recent times. In the last 46 years, over 26 outbreaks of H5N1 had been documented worldwide. This rapidly evolving pathogen of both veterinary and human health first emerged in 1996 from apparently healthy ducks in Southern China. It spread to over 60 countries in Eurasia, over 500 million poultry were culled, 505 human cases were recorded with over 300 mortality. It is a highly contagious viral disease in poultry with 100% morbidity and mortality in susceptible birds. This disease known as highly pathogenic avian influenza (HPAI), is listed as a notifiable disease by the Office International Des Epizootes (OIE) under List A disease. All HPAI viruses were considered to be of H5 or H7 haemagglutinin subtype.

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In order to isolate, amplify, clone and express H5, N1 and NS gene of AIV virus subtype H5NI, *Pichia pastoris* expression system was used. They were successfully cloned and expressed in the methylotrophic yeast *Pichia pastoris*. The haemagglutinin (H5HA) and nonstructural protein (NS1NS) recombinant proteins were generated using sticky ends ligation insertion of these genes into the multiple cloning sites of pPICZA and pPICZαA expression vectors, respectively. The inserted genes were confirmed by restriction enzymes analysis, polymerase chain reaction (PCR) and deoxyribonucleic acid DNA sequencing analysis. The recombinant plasmid construct were appropriately linearized and integrated into the chromosomal genome locus of *Pichia pastoris* by transformation through single cross over phenomena by electroporation.

Transformation efficiency were observed to be enhanced significantly ($P<0.05$) with more than 100 folds after pretreatment of *Pichia pastoris* competent cells with 0.1M Lithium acetate (LiAc) and 10mM Dithiothreitol (DTT). Other factors involved include concentration of linearized plasmid DNA, cell biomass density, cell growth phase and restriction enzymes used for the digestion of the plasmid. All these put together, significantly ($P<0.05$) enhances the transformation efficiency by electroporation using 1.5 Kv, 2.5 µF and 200 Ω with over a 100 fold difference with a minimum of 2.5×10^5 transformants/µg of DNA.

The recombinant H5 and NS1 proteins were expressed in methylotrophic yeast *Pichia pastoris* using shake flask high cell density fermentation as intracellular protein via pPICZA and as secretory protein via pPICZαA respectively. An optimum condition of 2.0% periodic methanol induction of 120 hrs and 216 hrs post induction time using

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complex media composition (YPTG/YPTM). An appropriate depression/repression switching of glycerol to methanol enriched complex media for the H5 and NS1 respectively, under the temperature of 25^{0}C at 250 rpm for ten successive days with 80% aeration at a pH 8.0 were observed to be optimal expression conditions.

The in vitro expressions of the fusion protein were confirmed by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. These analysis detected a molecular weight of full-length H5HA and NS1NS approximately ~ 88kDa and ~ 28kDa, respectively. There was a clear difference between the first 72 hrs and the subsequent hours post induction in H5HA expression dynamics while in respect of NS1NS the expression was evident at the 8th day post induction until the 10th day. In addition there was significant increase ($P<0.05$) in the growth of cell biomass density at repression/depression stage when simple minimum media and complex media were compared.

The study also revealed that the expressed recombinant protein was considered suitable as a potential diagnostic antigen for serological assay. In an in vitro diagnostic assay the recombinant protein expressed was applied as a coating antigen in an in-house developed preliminary ELISA assay for detection of avian influenza subtype H5N1, NS1 specific antibody. The results of the experiment showed that the known standard positive polyclonal sera (Abcam®, USA) reacted specifically with the purified recombinant NS1 protein as a coating antigen, thus, further confirming that the recombinant protein has properly folded and its antigenicity is maintained. Besides, it

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was also shown that the recombinant protein and the antibody are specific and homologous. When positive cell lysate and negative cell lysate based ELISA results were compared, more than three-fold difference was observed, which further reaffirmed the specificity and homologous nature of the recombinant protein. In addition preliminary ELISA assay based on the commercial antibody against the recombinant protein has showed a good correlation with an R^2 value= 0.972.

In summary, the current study has produced a recombinant H5 and NS1 recombinant protein in an in vitro, expression using single cell eukaryotic *Pichia pastoris* expression. This recombinant protein has demonstrated potential diagnostic values for avian influenza virus subtype H5N1: NS1 detection and identification. Recombinant plasmid transformation method has been remarkably enhanced via thiol compound pretreatment prior to electroporation. These new recombinant DNA technology could offer a niche for more efficient, safe, cheap and easy ways of generating in vitro diagnostic antigen as against the old traditional and cumbersome method of using whole viral particles antigens. Further investigation, understanding and application of this DNA technology could open up another niche into diagnostics and subunit vectored vaccine using *Pichia pastoris* in an effort to control the scourge of rapidly evolving epidemic of avian influenza.

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

PENCIRIAN MOLEKUL DAN PENGEKSPRESAN H5, N1 DAN NS1 PROTEIN REKOMBINAN SUBTIP VIRUS AVIAN INFLUENZA H5N1 DALAM *Pichia pastoris*

Oleh

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Subtip virus influenza unggas (AI) H5N1 adalah salah satu ancaman besar bagi industri unggas dengan implikasi kesihatan yang signifikan pada manusia di seluruh dunia. Ianya masih kekal penyakit unggas paling ditakuti pada masa sekarang. Dalam 50 tahun kebelakangan ini lebih dari 31 wabak H5N1 telah didokumentasikan diseluruh dunia. Patogen ini yang berkembang pesat dari segi kesihatan haiwan dan manusia, pertama kali muncul pada tahun 1996 dari itik yang kelihatan sihat di selatan China . Ianya tersebar ke lebih dari pada 60 negara di Eurasia, lebih dari 500 juta unggas dimusnahkan, 505 kes manusia dilaporkan dengan lebih daripada 300 kematian. Ini adalah penyakit virus yang sangat menular pada unggas dengan morbiditi 100% dan kematian pada unggas yang tiada imuniti. Penyakit ini, *highly pathogenic avian influenza* (HPAI) disenaraikan sebagai penyakit yang mesti dilaporkan oleh Pejabat Antarabangsa Dis Epizootes (OIE) di bawah penyakit senarai A. Semua virus HPAI dianggap H5 atau H7 subtip hemaglutinin.

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Dalam rangka untuk mengasingkan, memperbanyakkan, pengklonan dan mengungkapkan, gen H5, N1 dan NS virus subtip H5NI AIV, ekspresi *Pichia pastoris* digunakan dalam kajian ini. Gen berkenaan berjaya diklon dan diekspres dalam *methylotrophic* yis *Pichia pastoris*. Hemaglutinin (H5HA) dan struktur protein rekombinan (NS1NS) dihasilkan, dengan menggunakan hujung melekat penyisipan ligasi gen ini ke dalam kawasan pengklonan beberapa pPICZA dan vektor ekspresi pPICZαA masing-masing. Gen yang dimasukkan disahkan dengan analisis restriksi enzim, polimerase rangkaian bereaksi (PCR) dan asid deoksiribonukleik analisis DNA penjujukan. Mengambil kira semua analisis di atas, ianya mengesahkan klon yang berturutan gen putatif dalam rangka dengan vektor ekspresi dan menghasilkan BLASTN homologi 98-100% dengan InfluenzaA/Ayam/Malaysia/5858/2004/H5N1.

Konstruk plasmid rekombinan dilinierisasi dan diintegrasi sewajarnya ke dalam genom lokus kromosom dari *Pichia pastoris* dengan transformasi melalui fenomena silang tunggal dengan elektroporasi. Kecekapan transformasi dilihat dapat ditingkatkan secara signifikan ($P<0.05$) dengan lebih daripada 100 ganda selepas pre-rawatan *Pichia pastoris* sel kompeten dengan Lithium asetik 0.1 M (LiAc) dan 10mm Dithiothreoid (DTT). Faktor lain yang terlibat termasuk konsentrasi DNA plasmid *linearized*, sel kepadatan biojisim, sel fasa pertumbuhan dan sekatan enzim digunakan untuk pencernaan plasmid, ianya adalah signifikan ($P<0.05$) dalam konbinasi semua factor ini meningkatkan kecekapan transformasi dengan elektroporasi menggunakan 1.5 Kv, 2.5 μF dan 200 Ω, dengan lebih daripada perbezaan 100 kali ganda dengan minimum sebanyak $2.5x10^{5}$transforman/μgDNA.

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Rekombinan H5 dan NS1 protein diekspres dalam yis methylotrophic *Pichia pastoris* menggunakan *shake flask* fermentasi sel kerapatan tinggi sebagai protein intraselula melalui pPICZA dan sebagai protein sekresi melalui pPICZαA masing-masing. Keadaan optimum 2.0% induksi metanol berkala 120 jam dan 216 jam waktu pasca induksi menggunakan komposisi kompleks media (YPTG / YPTM) dengan kemelesetan yang sesuai / penindasan beralih dari gliserol ke media kompleks banyak metanol untuk H5 dan NS1 masing-masing, di bawah suhu 25^0C pada 250 rpm selama sepuluh hari berturut-turut dengan aerasi 80% pada pH 8.0, diamati sebagai ekspresi keadaan yang optimum.

Dalam ekspresi in vitro protein gabungan disahkan dengan natrium sulfat dodecyl elektroforesis gel akrilamida poli (SDS-PAGE) dan analisis Western blot. Analisis seperti ini mengesan berat molekul panjang penuh H5HA dan NS1NS sekitar ~ 88kDa dan ~ 28kDa masing-masing. Ada perbezaan yang jelas antara 72 jam pertama dan jam berikutnya pasca induksi dalam dinamik ekspresi H5HA, sementara berhubung NS1NS ekspresi tampak jelas di pasca induksi hari ke-8 hingga hari ke-10. Selain itu terjadi peningkatan yang signifikan ($P<0.05$) dalam pertumbuhan kepadatan sel biojisim pada penindasan / tahap kemelesetan ketika minimum media sederhana dan media kompleks dibandingkan.

Kajian ini juga mendedahkan bahawa protein rekombinan yang diekspres dianggap sesuai sebagai antigen diagnostik berpotensi untuk ujian serologi. Dalam esei diagnostik in vitro protein rekombinan yang diekspres digunakan sebagai antigen pelapis dalam esei ELISA awal yang dibangunkan secara dalaman, untuk mengesan subtip flu burung

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H5N1 NS1 antibodi spesifik. Keputusan kajian menunjukkan bahawa sera positif standard khas bertindak balas secara spesifik dengan protein NS1 rekombinan tulen sebagai antigen pelapis, dengan demikian, seterusnya mengesahkan bahawa protein rekombinan telah benar dilipat dan antigenisitinya adalah kekal. Selain itu juga ia menunjukkan bahawa protein rekombinan dan antibodi adalah spesifik dan homologi. Berdasarkan hasil keputusan ELISA, apabila sel lisat positif dan sel lisat negatif dibandingkan, lebih daripada tiga perbezaan didapati, yang selanjutnya mengesahkan sifat spesifik dan homologi protein rekombinan. Di samping itu, berdasarkan esei awal ELISA antibodi komersial terhadap protein rekombinan telah menunjukkan korelasi yang baik dengan nilai R2 = 0.972.

Sebagai rumusan, kajian ini telah menghasilkan H5 dan NS1 protein rekombinan dalam ekspresi in vitro, dengan menggunakan sel tunggal eukariotik *Pichia pastoris*. Rekombinan protein ini telah menunjukkan potensi nilai diagnostik bagi virus influenza unggas subtip H5N1: NS1 untuk pengesanan dan pengenalan. Kaedah transformasi plasmid rekombinan telah ditingkatkan pada kadar tinggi, melalui pre-rawatan sebatian tiol sebelum elektroporasi. Teknologi DNA rekombinan yang baru ini boleh dijadikan *niche* bagi cara yang lebih cekap, selamat, murah dan mudah dalam menghasilkan antigen in vitro diagnostic, dibandingkan kaedah praktikal tradisional yang lama dan susah yang menggunakan antigen penuh partikel virus. Kajian yang lebih banyak dalam pemahaman dan penerapan teknologi DNA ini akan membuka *niche* baru dalam pembangunan ujian diagnostik dan subunit vektor vaksin, melalui penggunaan *Pichia pastoris,* dalam usaha untuk mengawal pusingan cepat perubahan epidemik influenza unggas.

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I certify that a Thesis Examination Committee has met on September 2011 to conduct the final examination of Mustapha Bala Abubakar on his thesis entitled "Molecular Characterization and Expression of H5, N1 and NS1 Recombinant Proteins of Avian Influenza Virus subtype H5N1 in *Pichia pastoris*" 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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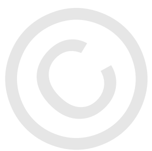
DECLARATION

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



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Date: 13th December 2011

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LIST OF ABBREVIATIONS

AIV	avian influenza virus
BCP	1-bromo-3-chloro-propane
BCIP	5-bromo-4-chloro-3-indolyl phosphate
β-gal	β-galactosidase
bp	base pair
cDNA	complementary deoxyribonucleic acid
C-terminus	carboxy terminus
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotides
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
h	hour
HA	haemagglutinin
His	histidine
HPAI	highly pathogenic avian influenza
kb	kilo base
kDa	kilo Dalton
LB	luria bertani
LiAc	lithium acetate
LPAI	low pathogenic avian influenza

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μg	microgram
μl	microlitre
μM	micromolar
mA	milliampere
mRNA	messenger RNA
NA	neuraminidase
NP	nucleocapsid protein
NS	non structural protein
nt	nucleotide
N-terminus	amino terminus
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
pH	*puissance hydrogene*
PCR	polymerase chain reaction
pPICZαA	extracellular vector
pPICZA	intracellular vector
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
s	second
SDS	sodium dodecyl sulphate

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TAE	tris-acetate – EDTA buffer
TBA	tris-buffered saline
Taq	thermus aquaticus
TEMED	tetramethyl ethylenediamine
U	unit
uv	ultraviolet
Vol	volume
w/v	weight/volume
YC	minimal medium for yeast
YPD	yeast peptone dextrose
YPDS	yeast peptone dextrose sorbitol
YPTM	yeast peptone tryptic soy broth methanol

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CHAPTER ONE

INTRODUCTION

Modern poultry industry has totally revolutionized the poultry production system in all ramifications ranging from tremendous increase in production capacity to improved bio-security, general health care, disease prevention, and control. This turn around began in the mid 1950's, before then poultry were kept as small backyard flocks reared under free range or semi-intensive system. With the modernized system, hundred(s) of thousands of birds can be kept under fully automated closed-house system. These closed-systems are often prone to crowding thereby limiting available supply of fresh breathing air as well as a common source of feed and water. Such conditions thus create conducive environment for proliferation and spread of pathogens within the flock and even beyond, to neighbouring farms. However, the modernization in farm practice itself is vital owing to the current challenges of global population expansion, which poses a serious threat to regional, national, and global food security. As projected by experts, by the year 2030 the anticipated world population would be 9.3 billion. This calls for a commensurate growth in food production for security and survival to curb against a global food crisis. Currently, the annual global poultry output stands at 35 – 40 billion chickens, 25% which comes from the USA; production of cheaper animal protein is thus one major challenge of the modern poultry system (Suarez *et al.*, 2006; Swayne, 2008; Webster, 2010) As part of the solution to the problem, there would be a need for an increased production of broiler, chicken, and eggs. While the modern poultry production is the answer, it remains a fact that it has its own inherent issues, challenges and shortcomings

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including: extremely rapid disease spread within and around the flock in the face of an outbreak, demand for high throughput and rapid diagnostic tools, new strategies for preventing and combating disease outbreak(s). Furthermore, this turn around revolution has cut across geographical boundaries, owing to the threat posed by the availablity of modern fast and efficient transport systems that easily facilitates human and pathogen haulage across the globe in a short time. This has a high impact on disease transmission/movement into and out of both human and animal population through the international trading activities of animal and animal products.

This global poultry revolution has positively influenced the current success of poultry industry in Malaysia. Poultry is the most successful sector of livestock that contribute more than 53% to the livestock industry in Malaysia. It provides income and livelihood source valued to the tune of over Ringgit Malaysia (RM) 5.468 billion annually with over 2,500 poultry farms producing more than 400 million birds per annum (GAIN Report, 2006). It has one of the highly world rated per capita consumption of animal protein (Chicken meat) of 32kg, and per capita egg consumption level of 280 eggs per person per annum. The country is 100% self sufficient in meeting its demand for animal protein with poultry contributing 95% of the overall meat and eggs produced. The livestock industry recorded a remarkable and successful annual growth of 5.6% for the last decade (2000 – 2009) with production increasing from 714,300 to 1.202 million metric tones, with ex-farm value of RM5.468 billion or 53% of ex-farm value of the total livestock industry. The total export of chicken products increased from RM54,44 million in 2007 to RM350.68 million in 2009. More so, 510 million metric tones of eggs was produced in 2009 with the total ex-farm value of RM 2.226 billion, contributing

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22% of the ex-farm value of the livestock industry (GAIN Report, 2006; GAIN Report, 2005; Idris, 2010) .

This evolution in the Malaysian poultry industry from mere subsistent farming to a commercialized and advanced industry took a course for the past 60 years. It operates on a similar platform and pattern with the modern trend found around the world, through introduction of superior breeds, vaccines for disease control, in addition to the government supported favourable policies put in place (Aini, 2005).

While it has been clearly shown that poultry industry has significantly contributed to the realization of Agriculture as the third Malaysian engine of growth, development, and creation of revenue generation and provision of sustainable food security. The strategic role played by poultry in the country's mainstream economy now faces threats posed by the spate of emergence and re-emergence of infectious diseases like any other country in the world, since some of these infectious diseases do not have respect for geographical boundaries. These include avian influenza, Newcastle disease, infectious bursal disease, infectious bronchitis, and chicken anaemia virus.

Avian influenza (AI) subtype H5N1 is one of the major threats to poultry production and human health implication across the World; it is the most dreaded poultry disease in recent times caused by an Orthomyxovirus. H5N1 is an important veterinary and human health pathogen that was first emerged in 1996 from apparently health ducks in Southern China. It has spread to over 60 countries in Eurasia, with over 500 million poultry destroyed, 505 human cases were recorded with 300 mortality. It is a highly contagious viral disease of poultry with 100% morbidity and mortality in susceptible birds (Capua and Alexander, 2007; Suarez *et al.*, 2006; Swayne, 2008a; Webster, 2010). This disease

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(HPAI) is listed as a notifiable disease by the Office International Des Epizootes (OIE) under List A Disease. Within the OIE code for international trade, trading restrictions and embargoes are placed to prevent the introduction of foreign poultry diseases such as highly pathogenic notifiable avian influenza (HPAI) by live birds, poultry meat, and other poultry products to countries or regions free of HPAI.

In the past 48 years, 26 epidemics or limited/sporadic outbreaks of highly pathogenic avian influenza (HPAI) have been documented worldwide with the aid of consistent diagnostics and control strategies in place. All of these HPAI viruses were found to be of H5 or H7 haemagglutinin subtypes (Swayne, 2008; Hugita,2007; FAO, 2006; FAO/OIE 2005) . The number of epizootics, cases (i.e., farms), and number of birds affected by HPAI has grown geometrically since 1959. From 1959-1998, the number of birds affected with HPAI outbreaks stands at 23 million, while from 1999 to early 2004 it increased by more than 10 folds with over 200 million birds involved (Capua, 2004). From 2007, with the completion of outbreaks in Canada and North Korea, the outbreaks spread further to more countries in Asia, Europe, and Africa, the number of dead and culled birds now exceeds 500 million. The H5N1 HPAI that appeared in 1996/97 epidemic was the largest outbreak recorded in 50 years, exceeding 300 million birds either affected by the disease or culled. This epidemic has spread from its initial country of report in Southern China in 1996 to affect poultry and wild birds in over 60 countries in Africa and Eurasia. In spite of the fact that some few countries were able to successfully eradicate H5N1, the basic facts are that: [1]. the establishment of multiple epicenter of the virus in wild birds, village poultry and live poultry market (LPM) obviously evidenced in many countries (especially domestic ducks which occasionally show disease) is worrisome. [2]. lack of control of movement of village poultry and

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existence of LPM systems. Also the inapparent infection of migratory waterfowls has created a favorable medium for recurrence of disease outbreaks within countries and as well facilitate viral reintroduction into countries that were declared free of HPAI in 2004 and 2005 such as Japan, South Korea, and Thailand, from late 2006 to early 2007 (Hugita, 2007; Kim, 2006; Swayne, 2008a; Swayne and Halvorson, 2008). The economic impact of HPAI was reflected clearly in these outbreaks /epidemics.

In Malaysia, the history of Avian Influenza Virus (AIV) started with the isolation of only LPAI subtypes H4N3, H4N6, H3N6 and H9N2 in domestic duck (Aini and Ibrahim, 1986). However, in the beginning of August 2004, a LPAI virus, subtype H5N2 was identified in ducks exported from a farm in Perak state, Malaysia to Singapore (Aini and Ibrahim, 1986).The first case of the HPAI virus subtype H5N1 was in two free-range chicken flocks of approximately sixty birds located in the state of Kelantan, Malaysia boardering Thailand. It was reported on the 19th August, 2004 (Sabirovic *et al.*, 2004).The 2004 outbreaks of same H5N1 in the neighboring countries of Vietnam and Thailand were highly fatal to human and poultry.

Malaysia was fortunate enough, that despite HPAI outbreaks still occurring in some of the neighboring ASEAN countries, it managed to eradicate the disease successfully on three occasions. The first, second and third wave of HPAI outbreaks occurred in August 2004, February 2006 and June 2007, respectively. Freedom from the disease was regained in May 2005, June 2006, and September 2007, respectively. The dates of freedom declaration (DFODFD), of the three outbreaks, were 276, 124 and 95 days respectively. Birds culled during each wave were 15,537; 58,457 and 4,266,

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respectively. The cost of handling the disease was really outrageous in spite of the short span of the outbreak, a total of RM 5.7 million, RM 3.1million and RM 0.58 million, respectively were spent on these three outbreaks to eradicate the disease successfully (DVS/VRI, 2009).

In spite of the effort and support by the Malaysian authority in eradicating HPAI, there is still the need for research efforts to come up with a more definitive and effective control and prevention strategy. Also in need is a rapid, sensitive and specific diagnostic tool that will ensure prompt diagnosis of the disease and perhaps differentiating infected bird from vaccinated for the sake of International trade regulation and embargo. This effort will further ensure protection of the country from introduction of exotic or foreign poultry diseases such as HPAI through an effective national active and passive surveillance program campaign.

The first pandemic influenza of the twenty first (21st) century that originated from swine in Mexico in April 2009 has clearly demonstrated the significance of recombinant DNA technology in the efforts to efficiently prevent and control emergence disease in the face of a global outbreak in both human and lower animals using modern and new generation vaccine development strategies. The pandemic H1N1 2009 spread globally in human in a remarkably short time and has a wide zonootic host range including swine, turkey, ferrets, cats, and dogs. There was an urgent need for rapid vaccine production of both inactivated and live attenuated influenza vaccine that replicated well in embryonated chicken eggs and cell cultures.

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Reverse genetics and conventional reassortment were used to generate high yielding vaccines strains. Other strategies for rapidly producing H1N1 to generate vaccines included in vitro expression of haemagglutinin in baculovirus. While approved influenza vaccines for humans currently include only whole virus, subunit and live attenuated vaccines many recombinant strategies including single cell eukaryotic vector based recombinant are undergoing development and some of these are approved for use in poultry. Vaccines and diagnostics antigen for animal including domestic poultry have been developed for the HPAI H5N1 avian influenza virus "bird flu" that is a threat to both human and veterinary public health in multiple epicenter in Eurasia (Webster, 2010). The strategies for vaccine and diagnostics development and use have been widely used in Eurasia specifically in China, Vietnam, Indonesia, and Egypt. The strategies include [1]- Inactivated oil emulsion vaccines [2]- In vitro expression system including Baculovirus in insect culture, Vaccinia, and Alpha virus: virus like replicon particle [3]- In vivo expression system including live attenuated influenza virus, Fowl pox, Avian leukosis virus, Paramyxovirus type 1 vectored, Gallid herpesvirus-1 and DNA vaccine-Naked DNA.

In the present study, successes and failures, advantages and disadvantages of using single cell eukaryotic vector as a backbone for the expression of AIV H5, N1 and NS1 genes in *Pichia pastoris* recombinant protein will be assessed. This is in the light of its use in potential diagnostics for HPAI A/Chicken/ Malaysia /2004(H5N1) as part of the effort for the control and prevention of HPAI in Asian poultry. Additionally attempts would be made to address the problem of inability to differentiate infected birds from vaccinated (DIVA) birds. The advent of recombinant DNA technologies has further improved upon the existing benefits of the conventional diagnostics methods and

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vaccines. In comparison to conventional ELISA assays which employed the use of whole viral particles, the present ELISA kits will involve the use of recombinant protein for specific genes. This offers increased sensitivity, specificity and rapidity in detection of Malaysian locally existing/isolated strains and subtype H5N1 which hitherto does not exist. This also could have a potential for differentiating of infected birds from vaccinated (DIVA) using the novel concept of DIVA. On the other hand, the recombinant antigen will serve as a bedrock/ foundation for developing a recombinant vaccine. In an attempt to develop a more efficient production of this recombinant protein as a potential diagnostic antigen and perhaps a suitable vaccine candidate, *Pichia pastoris* was investigated as a potential expression system. This methylotrophic yeast has been successfully used for functional expression and secretion of a broad spectrum of a proteins (Cereghino and Cregg, 2000a).

It is hypothesized that the ability to insert H5 haemagglutinin, N1 neuraminidase and NS1 non-structural protein genes into the DNA genome of *Pichia pastoris* via pPICZA and pPICZαA expression vectors may enable the production of recombinant protein, expressing the foreign avian influenza virus H5HA, N1NA and NS1NS genes, which could also have a potential diagnostic value. Expression of viral antigens in a recombinant *Pichia pastoris* given, the genomic characteristic of *Pichia pastoris* makes could be feasible.

Attempts have been made to generate a recombinant *Pichia pastoris* capable of expressing avian influenza virus subtype H5N1: H5, N1 and NS1 protein, with the goal of producing a recombinant protein in *Pichia pastoris* with diagnostic values and potential.The study focused mainly on the cloning, expression, and purification of avian influenza virus subtype H5N1, H5, N1, and NS1 genes recombinant protein in a *Pichia*

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pastoris, as well as evaluation of the immunogenicity of the expressed proteins in vitro and in vivo.

The ability to clone and express H5, N1 and NS1 proteins into the DNA genome of *Pichia pastoris* may enable the development of suitable recombinant protein with a diagnostic value and potentials (recombinant *Pichia pastoris)* for effective and efficient control of HPAI.

The specific objectives of this study were:

1. to amplify and clone avian influenza virus gene encoding H5, N1 and NS1 proteins into *Pichia* expression vectors.
2. to enhance the transformation efficiency of *Pichia* plasmid.
3. to express H5, N1 and NS1 recombinant proteins of AIV subtype H5N1 in *Pichia pastoris*.
4. to determine the diagnostic potential of NS1 recombinant protein.

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