Development of Vaccine Approaches for Pandemic Influenza Infections

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged; and ethics procedures and guidelines have been followed.

Yu-Chen Lin
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<thead>
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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Δ</td>
<td>Deletion</td>
</tr>
<tr>
<td>λ</td>
<td>Lambda phage DNA</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μL</td>
<td>Microlitre</td>
</tr>
<tr>
<td>μF</td>
<td>Microfarad</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>Ω</td>
<td>Ohm</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AIV</td>
<td>Avian influenza virus</td>
</tr>
<tr>
<td>Alum</td>
<td>Aluminium-salt-based adjuvant</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AT</td>
<td>Autotransporter</td>
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<tr>
<td>bp</td>
<td>Base pairs</td>
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<tr>
<td>BCIP/NBT</td>
<td>5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt/ nitro-blue tetrazolium chloride</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
</tr>
<tr>
<td>BMMH</td>
<td>Buffered minimal glycerol medium</td>
</tr>
<tr>
<td>BMMY</td>
<td>Buffered methanol-complex medium</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxyl terminus domain of an amino acid sequence/protein</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CMI</td>
<td>Cell-mediated immunity</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>CV</td>
<td>Column volume of resin used in chromatography</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
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<td>Da</td>
<td>Dalton</td>
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</table>
DC  Dendritic cell
DMEM  Dulbecco's modified Eagle's medium
DMSO  Dimethyl sulfoxide
DNA  Deoxyribonucleic acid
DNase  Deoxyribonuclease
DPBS  Dulbecco's Phosphate-Buffered Saline
EDTA  Ethylenediamine tetra acetic acid
ELISA  Enzyme-linked immunosorbent assay
ELISpot  Enzyme-Linked Immunospot assay
EtBr  Ethidium bromide
FA  Freund adjuvant
FBS  Fetal bovine serum
FCS  Fetal calf serum
FCA  Freund's complete adjuvant
FIA  Freund's incomplete adjuvant
g  Gram
x g  Gravitational acceleration
H₂O  Water
H₂O₂  Hydrogen peroxide
HA  Influenza hemagglutinin
HA1  HA domain 1
HA2  HA ectodomain (domain 2)
HA unit  Hemagglutination unit
HBV  Hepatitis B virus
HCl  Hydrochloric acid
HI unit  Hemagglutination inhibition unit
HPAIV  Highly pathogenic AIV
HPV  Human papillomavirus
HRP  Horseradish peroxidase
i.m.  Intramuscular
i.p.  Intraperitoneal
IBT  Institute of Biotechnology, Vietnam Academy of Science and Technology
IFN-γ  Interferon gamma
Ig  Immunoglobulin
IL-1β  Interleukin 1 beta
IL-12β  Interleukin 12 beta
IL-15  Interleukin 15
IL-18  Interleukin 18
IL-2  Interleukin 2
IL-4  Interleukin 4
IMAC  Immobilised metal affinity chromatography
IPTG  Isopropyl-β-D-thiogalactopyranoside
kbp  Kilobase pairs
kDa  One thousand Daltons
kV  One thousand Volts
L  Litre
LB  Luria-Bertani
LD₅₀  The 50% lethal dose, the concentration required to kill 50% of a population
LN  mucosa-draining lymph node
LPAIV  Low pathogenic AIV
LPS  Lipopolysacharide
LT  Heat-labile toxin
mg  Milligram
mH₂O  Milli Q water
min  minute
mM  Millimolar
mol  Mole
mRNA  Messenger RNA
M  Molarity
M1  Influenza matrix protein 1
M2  Influenza matrix protein 2
MALT  Mucosa- associated lymphoid tissue
MCA  MacConkey agar
MCS  Multiple cloning site
MDCK  Madin-Darby canine kidney cell
MDH  Minimal dextrose medium with histidine
MH  Mueller-Hinton
MHC  Histocompatibility complex
MMH  Minimal methanol medium with histidine
MVA  Vaccinia virus Ankara
MW  Molecular weight
ng  Nanograms
nm  nanometer
nM  Nanomolar
N₂  Nitrogen
N-terminus  Amino terminus domain of an amino acid sequence/protein
NaCl  Sodium chloride
NaOH  Sodium hydroxide
NA Neuraminidase
NALT Nasopharynx-associated lymphoid tissue
NB Nutrient broth
NCS New born calf serum
NEP influenza nuclear export protein
NP Influenza nucleoprotein
NS1 Influenza non-structural protein
OD Optical density
OM Outer membrane
ORF Open reading frame
pH Negative algorithm of hydrogen ion concentration
pmol picomole
PA Influenza Polymerase acidic protein
PAGE Polyacrylamide gel electrophoresis
PAMP Pathogen-associated molecular patterns
PBS Phosphate buffered saline
PBST PBS supplemented with 0.05% (v/v) Tween 20
PCR Polymerase chain reaction
PD Passenger domain
PIC Protease Inhibitor Cocktail
PR-8 Influenza strain H1N1 (A/influenza/Puerto Rico/8/34)
$PstI/\lambda$-DNA Lambda phage DNA digested with the restriction enzyme $PstI$
rpm Rotation per minute
rHA1 Recombinant HA1
R Resistance to antimicrobial
RE Restriction enzyme
RNA Ribonucleic acid
RNase Ribonuclease
RT Room temperature
s Second
s.c. Subcutaneous
spp Species (plural)
S.D. Standard deviation
SDS Sodium dodecyl sulphate
SFC Spot forming unit
SP Signal peptide
STM1 $Salmonella$ Typhimurium aroA' vaccine strain
t.c. Transcutaneous
TB Tuberculosis
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethyl ethylenediamine</td>
</tr>
<tr>
<td>Th₁</td>
<td>Effector T-cells involved in the initiation of cell-mediated immune responses</td>
</tr>
<tr>
<td>Th₂</td>
<td>Effector T-cells involved in the initiation of humoral immune responses</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Tm°C</td>
<td>Melting temperature</td>
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<tr>
<td>TMB</td>
<td>3, 3’, 5, 5’-tetramethylbenzidene</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) amino methane</td>
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<tr>
<td>V</td>
<td>Voltage</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>YNB</td>
<td>Yeast Nitrogen Base</td>
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Abstract

Highly pathogenic avian influenza virus is a continuous threat to the global poultry industry, and results in occasional spill-over infections into the human population. The rapid antigenic shift and drift of influenza virus often results in outbreaks of novel influenza virus, such as the highly pathogenic H5N1 avian flu. The virus has broad reservoirs from sea mammals to avian species, and thus eradication of the virus has proven difficult, therefore the prevention of virus spread and circulation is considered the most effective method for disease control [1].

The first objective of this study was to evaluate the immunogenicity of a recombinant influenza haemagglutinin domain 1 (rHA1) expressed in *Pichia pastoris* (*P. pastoris*). In a project previously carried out in my Master’s study, the HA1 genes derived from influenza H1N1 (A/Puerto Rico/8/34) was cloned in *P. pastoris* vector, and the rHA1 was successfully expressed as soluble secreted protein, and purified using Immobilised metal ion chromatography (IMAC) under native conditions. The rHA1 protein was administered into mice to evaluate its immunogenicity. The animal study has revealed that, immunised mice exhibited robust Th2-type T-lymphocyte and anti-HA1 IgG responses, and developed a virus-neutralising antibody response for hemagglutination inhibition (HI) at titer 7.4 log₂. The result suggests that the *Pichia*-expressed rHA1 may be used as an immunogen for the prevention of influenza infection, and the technique developed in this study could serve as a cost and time effective solution for influenza virus vaccines.

*Salmonella* have many advantages as a vaccine delivery vector, as they are easy to produce, easy to administer (orally), and are able to elicit both humoral immunity including serum and secretory IgA antibody, and strong cell mediated immune responses that include cytotoxic
and memory T-lymphocytes [2-4]. Furthermore, vaccinated recombinant Salmonella not only elicit immune responses against the heterologous antigen, but also to Salmonella itself, providing protective immunity against both Salmonella and the heterologous pathogen.

The second objective of this study was to use Salmonella Typhimurium strain STM1 (an ΔaroA mutant developed at RMIT), as a delivery vector for an influenza antigen. Different strategies were used to present the influenza hemagglutinin (HA) antigen from various destinations to optimise immunogenicity. These include one to display the HA antigen on the outer membrane of Salmonella (utilising plasmid pHES, encoding the N-terminal signal peptide and the essential C-terminal translocation unit of the ShdA autotransporter from S. Typhimurium strain LT-2 [5]) and another to secrete it into the media (pMOhly1, encoding the necessary components of the E. coli α-haemolysin secretion system [6]).

After successful cloned the HA1 gene into each plasmid vector and passed into STM1, the expression was characterised. Protein analysis indicated that rHA1 expressed using pHES vector was translocated to the outer membrane fraction of the STM1 surface; whereas rHA1 expressed using pMOhly1 vector was detected in the growth medium. A chicken study was carried out to evaluate the immunogenicity of these STM1 clones; the vaccine strains were administered through oral gavaging and STM1 harbouring empty pHES and pMOhly1 plasmids were used as control group in the study.

The animal trial results indicated that STM1 expressing HA1 using pHES were unable to persist/colonise after administration, and neither humoral nor cell-mediated immune responses against influenza HA1 were elicited using the vaccine strain. On the other hand, STM1 expressing HA1 using pMOhly1 was able to induce a Th1-type immune response, indicated by the secretion of INF-γ from splenocytes after being stimulated by either
Pichia-expressed rHA1 or inactivated influenza PR-8 virus. However, humoral immune responses were not elicited using this vaccine.

Taken together, these results indicated that Pichia-expressed influenza rHA1 could induce not only virus-specific humoral immune responses, but also stimulate a Th2-type cell-mediated immune response, which would likely be protective against influenza infections. The STM1 expressing secretory rHA1 using pMOhly1 vector successfully induced Th1-biased cell-mediated immune responses, which are suggested to be important in delayed-type viral clearance [7].

Even though the results acquired in these studies were not optimal, there were significant responses to the influenza antigen which suggested that further investigation is required. For this to happen, it is possible to induce a more well-balanced immune response against influenza virus by combining both subunit rHA1 and STM1 vaccine strain into a prime-boost vaccine scheme. Such a vaccine regimen could ensure the elicitation of both humoral and cell-mediated immune responses against influenza infections.
Chapter 1

Literature Review
1.1 Introduction

The prevention of infectious diseases has been one of the most difficult tasks for humanity. The burden associated with disease and infection is diverse and inevitable. Before the era of vaccination, diseases and infections were often managed by improved education, hygiene, and drug treatment. However, in terms of disease management, it is always more beneficial for the public to prevent diseases rather than to treat and cure them. The prevention of disease is often done by immunisation, otherwise known as vaccination, which modulates the host immune system to inhibit disease progression. Currently, there are many successful vaccines available for the prevention of a variety of diseases in humans and animals, but there are still diseases that kill millions of lives globally which urgently demand a vaccine. Vaccine development is dependent on the understanding of disease etiology; the immune responses associated with the disease and most importantly, to sustain long-lasting immunity for ongoing protection. This review will discuss the evolution of vaccines from its first documented practice to the more advanced novel vaccines, and the modern achievements used in the prevention of influenza infections.

1.2 Evolution of Vaccine Development

Variolation is the most primitive procedure of disease control in recorded history. The procedure involves the deliberate inoculation of infectious material such as purulent discharge, saliva or blood from an infected person into a healthy individual via direct contact [8]. The procedure is believed to have originated in India or China, but was introduced to the United Kingdom in 1721 by Lady Wortley-Montagu, who was the wife of a British ambassador to the Sublime Porte. Despite the initial skepticism, variolation slowly became regular practice in the United Kingdom, and in 1757, an 8-year-old boy named Edward Jenner was variolated with smallpox. The procedure was a success, as the boy became immune to smallpox after recovering from a mild case of the disease. Jenner later became
well-known around the world for his innovative contribution in immunisation against smallpox using matter taken from cowpox lesions [9]. Variolation ultimately spared countless lives from smallpox - the deadliest disease of the time. Despite its great success, there were several risks associated within the procedure: 1) the procedure could weaken or even kill the recipient if incorrectly administered, or if the recipient was physically unfit to withstand the infection, and 2) the recipient could became contagious after variolation, risking an epidemic [10, 11].

Jenner’s pioneering work of infecting people with cowpox to confer protective immunity to smallpox was widely considered as the basis of modern immunology. Since then, the term vaccine has been used for any preparation of dead or attenuated pathogens or their products, where these preparations when introduced into the body conferred the elicitation of protective immune responses without causing disease. The next huge leap in regards to the understanding of infectious disease and immunity was achieved by the separate work of Louis Pasteur and Robert Koch and germ theory.

Louis Pasteur demonstrated that virulence of Pasteurella multocida could be attenuated and evidenced the concept of immunity and generalised the principle of vaccination [12], while Robert Koch developed general microbiology methods, diagnosis of tuberculosis, and public control measures for cholera. Together with his fellow scientist Jacob Henle, they compiled the Koch-Henle postulate for the definition of microbial pathogens [13, 14]. After Pasteur’s vaccine studies, Salmon and Smith developed the first inactivated whole-cell vaccine for the prevention of Salmonella infections in 1886 [10].

The field of vaccine development is still rapidly evolving three centuries after Jenner’s pioneering discovery. Over the centuries, vaccines have achieved many great successes,
including the eradication of smallpox, the close eradication of polio, and the prevention of many other infectious diseases with significant morbidities and mortalities [15]. However, there are still a considerable number of diseases which require an effective vaccine such as AIDS, tuberculosis, and malaria. Recent advances in molecular immunology, structure and computational biology, nanotechnology, and vaccine formulations have heralded in a new era of vaccine discovery in the 21st century [15].

1.3 Vaccine Delivery

Vaccine delivery is a crucial part in vaccine development, which encompasses: the route of vaccine administration and specific vaccine formulation for the activation of appropriate immune response [16]. Vaccine formulations can be administered to different sites of the body by intramuscular (i.m.), subcutaneous (s.c.), transcutaneous (t.c.), oral and nasal delivery. Each of these administration routes would require different vaccine formulations to optimise their immunogenicity [16]. Furthermore, vaccine dosage and number of vaccinations are also crucial to the successful elicitation of protective immune responses. Prior to licensing, these factors have to be carefully considered and evaluated in a randomised trial, which examines the vaccine efficacy at the individual level [17]. In general, the vaccine dosage should be kept to a minimum, but still sufficient to elicit a protective immune response in the majority of the test subjects. This could reduce the possibility of adverse effects caused by vaccine overdose [18-20], and increase the cost-effectiveness [17]. On the other hand, protective immunity following a single vaccine dosage can sometimes be incomplete and/or short-lived. Multiple doses of vaccines can be used to overcome these obstacle, which could improve the percentage of vaccinees who develop protective immune responses and prolong the duration of such immunity [21].
1.3.1 Intramuscular and subcutaneous administration

Subcutaneous administration is the oldest method for delivering vaccine, whereas intramuscular vaccination is a relatively new technique and is commonly used for modern vaccines such as Gardasil® and the seasonal influenza vaccine. Although i.m. vaccinations are routinely used in developed countries, there are several drawbacks in regards to this method of delivery which include: 1) trained personnel are required to administer the vaccines, 2) possible needle injuries 3) biohazardous waste – needle and syringe, 4) risk of bloodborne pathogen transmission, 5) cold storage and transportation for liquid-based vaccines 6) needle phobia, and 7) unable to induce mucosal immunity [16].

1.3.2 Intranasal and oral administration

In general, mucosal vaccine delivery is advantageous over i.m. and s.c. vaccinations as it eliminates risks associate with needle injections, and is more likely to induce both systemic and mucosal immune responses. Furthermore, mass vaccinations in Third World countries are more likely to be achieved [22-24]. Region-specific mucosa-associated lymphoid tissue (MALT) consists of inductive and effector sites, and mucosa-draining lymph nodes (LNs), and these specific mucosal inductive sites are crucial for eliciting both humoral and cell-mediated immune (CMI) responses [25-27]. Intranasal vaccines are usually administered by spraying, which allows a large contact surface of the nasal mucosa underlined with nasopharynx-associated lymphoid tissue (NALT), to be stimulated [22, 23]. One of the well-known intranasal vaccines is FluMist®, which is a trivalent live attenuated influenza vaccine given by nasal spray and licensed in the U.S. for persons aged between 5 ~ 50 years [28]. A large clinical study concluded that intranasal delivery was well tolerated, and after two doses of vaccine, 61% of initially seronegative children had antibodies to influenza A (H1N1), and 96% had antibodies to each of the other vaccine subtypes (A (N3N2) and Type B) [28]. However, there is an increased risk of developing Bell’s palsy, a paralysis or weakness
on one side of the facial muscles, associated with this vaccine [29]. Furthermore, the vaccine recipient may experience an unpleasant taste and nasal discomfort, which could discourage repeated use [30].

Oral vaccination inherits the advantages of mucosal vaccines. However, the development of oral vaccines is complicated by the fact that vaccines must be able to withstand the harsh conditions of the gastrointestinal tract [24]. Antigen uptake by intestinal epithelial cells and antigen presenting cells (APCs) is inefficient, which often leads to limited immunogenic properties [31]. However, a wide variety of antigen delivery particulates are under investigation to overcome these obstacles, including polymer- or nano-based microparticles, immun-stimulating complexes and liposomes [24, 32-35].

1.4 Adjuvants

Adjuvants are broadly defined as compounds that enhance the potency and longevity of a specific immune response to vaccine antigens. This can potentially make an ineffective vaccine become effective and has also been known as the “dirty little secret of immunologists” [36, 37].

Modern vaccines generally require the assistance of appropriate adjuvants to induce potent and long-lasting, specific immune responses to vaccine antigens [38]. Currently, there are three main types of vaccines: live-attenuated, inactivated, and subunit vaccines. Unlike live-attenuated vaccines, inactivated and subunit vaccines are usually less immunogenic, hence assistance from adjuvant is often required for enhancing, sustaining and directing vaccine immunogenicity [39, 40].
A good adjuvant or adjuvant formulation should be: 1) non-toxic or have minimal toxicity at the dosage to induce effective adjuvanticity, 2) capable of eliciting the desired humoral and/or cell-mediated immunity, 3) capable of conferring long-lasting immunity, 4) safe, without causing disease such as autoimmunity and cancer, and 5) assist in vaccine stability [37].

Modern adjuvants can be generally classified into two different categories according to their mechanism of adjuvanticity – immunostimulants and vehicles [38]. The functions of immunostimulants are to potentiate the specific immune response by directing an antigen to immune cells, facilitating phagocytosis and/or activating APCs [41]. Such a system can be achieved by formulating vaccine antigens together with naturally occurring immunostimulating proteins such as the *E. coli* heat-labile toxin (LT) or cholera toxin (CT) [42]. Other Immunostimulants such as lipopolysaccharides (LPS) derived from the bacterial outer membrane of gram-negative bacteria may be directly recognised by toll-like receptors (TLRs) on APCs, and initiate innate immune responses [35, 43].

Vehicle adjuvants, also known as the “depot delivery system”, are used to slow the release of vaccine antigens from the site of injection, hence optimising vaccine immunogenicity [39, 44]. This is often accomplished by entrapping the vaccine antigen in a poorly metabolised and slow-degrading compound such as aluminium-salt-based adjuvant (Alum) [38, 41]. Alum has been the most commonly used adjuvant. It was first published in 1929, and it’s the only adjuvant that is currently licensed for the use in human vaccines [45, 46]. Alum promotes antigen phagocytosis via APCs such as dendritic cells (DCs), macrophages and B-lymphocytes, and it also enhances MHC class II expression and antigen presentation [47, 48]. However, Alum is unable to elicit Th₁ and cytotoxic T-lymphocyte (CTL) responses, which are crucial in controlling most intracellular pathogens such as TB, malaria and AIDS [38]. Moreover,
formation of granulomas due to repeated injection of Alum-adjuvanted vaccine is also of concern [39].

1.5 Traditional vaccines

The traditional approaches to vaccine development have focused on using attenuated, inactivated or modified pathogens. There are numerous successful vaccines developed using these approaches (as summarised in Table 1.1). The traditional vaccines can be generally classified into three types: 1) attenuated vaccines; 2) inactivated vaccines; and 3) subunit vaccines (Figure 1.1).
Table 1.1: Examples of conventional vaccines

<table>
<thead>
<tr>
<th>Type of vaccine</th>
<th>Target pathogens</th>
<th>Type of immunity</th>
</tr>
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<tbody>
<tr>
<td><strong>Attenuated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial</td>
<td><em>Salmonella</em>, Tuberculosis</td>
<td>IgA, CMI</td>
</tr>
<tr>
<td>Viral</td>
<td>Measles, Mumps, Rubella, Polio</td>
<td>IgA, CMI</td>
</tr>
<tr>
<td><strong>Inactivated</strong></td>
<td></td>
<td></td>
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<tr>
<td>Bacterial</td>
<td><em>Salmonella</em>, <em>Yersinia</em>, Leptospires</td>
<td>IgG</td>
</tr>
<tr>
<td>Viral</td>
<td>Polio, influenza</td>
<td>IgG</td>
</tr>
<tr>
<td><strong>Sub-unit</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial</td>
<td>Clostridial Fusiformis-toxoid</td>
<td>IgG</td>
</tr>
<tr>
<td>Viral</td>
<td>FMDV-capsid</td>
<td>IgG</td>
</tr>
</tbody>
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Table adapted from [49]
Figure 1.1: Traditional vaccine methodologies.

Traditional vaccines were designed empirically using: a) Killed (inactivated) pathogen; b) attenuated pathogen; and c) subunit of target pathogen. Many successful vaccines were developed using these approaches. Image adapted from [50]
1.5.1 Attenuated vaccines

Upon attenuation, a pathogen is subjected to altered growth temperature, serial passage in cell-lines or cultures, or genetic modification. These procedures are performed to reduce or eliminate virulence, but also retain their ability to replicate at a reduced rate. As the live pathogen is still capable of establishing infection in target cells, they are able to induce “targeted” immunity against the pathogen. Furthermore, attenuated vaccines usually have the advantage of convenient administration, such as via intranasal or oral routes [51]. The use of attenuated vaccines is unsafe in immunocompromised individuals as the vaccine pathogen may persist due to a limited immune response, and reversion of these vaccine strains to the pathogenic wild-type is also of great concern [52, 53]. Moreover, attenuated vaccines have evolved into vaccine carriers, in which heterologous antigens from unrelated pathogens can be genetically engineered to be expressed in these carriers [3, 54]. Details of vaccine carriers will be discussed subsequently.

1.5.2 Inactivated vaccines

In contrast to live attenuated vaccines, inactivated vaccines are generally more stable and safe, as the pathogen has lost the ability to replicate by means of treatment with heat, chemical or radiation. However, the drawback of these vaccines is their inability to infect cells and subsequent lack of cell-mediated immunity, which makes the vaccines less immunogenic. Therefore, multiple injections and strong adjuvants are often required to elicit sufficient protective immunity [55]. Repeated vaccination with adjuvanted vaccines could potentially cause serious adverse reactions such as autoimmune disorders and allergy [56]. Despite these disadvantages and safety concerns, many inactivated vaccines are still commonly used today, such as the seasonal influenza vaccine and the inactivated polio vaccine [49].
1.5.3 Subunit vaccines

A subunit vaccine relies on the use of microbial components to elicit appropriate immune responses against the target pathogen. Since potentially harmful pathogen components are not administered, it significantly improves vaccine safety [57]. Subunit vaccines are easily manufactured in bulk quantities with high consistency, able to induce immune responses against specific pathogen epitopes, and are highly stable [58], but are generally less immunogenic than the traditional whole-cell preparations [59]. To ensure long-lasting protective immunity, the incorporation of potent adjuvants along with multiple administrations of the vaccine is necessary [57, 60]. A successful example of a subunit vaccine is the hepatitis B virus (HBV) vaccine which consists of highly purified recombinant envelope protein found on the viral surface [61, 62]. The immunisation strategy is a course of three injections which confers more than 95% and 90% protection in infants and adults, respectively [61]. However, non-responders to the HBV vaccine account for a small yet significant amount of vaccinees [63].

1.6 Modern vaccines

Following the era of traditional approaches in vaccine development, more sophisticated vaccines have been developed.

1.6.1 Conjugate vaccines

Conjugate vaccines use molecular technology to join a weak antigen such as bacterial polysaccharide - generally only possessing a B-lymphocyte epitope, to a carrier protein which contains T-lymphocyte epitopes to boost immunogenicity [64, 65].
1.6.2 **Synthetic peptides**

Synthetic peptides are an epitopes-based approach by synthesising small peptides containing regions of T-lymphocyte epitopes, and used as vaccine antigen to elicit potent cell-mediated immunity [66]. However, these peptides are often not as immunogenic as the whole protein, hence need to be optimised to enhance immunogenicity.

1.6.3 **Reverse vaccinology**

Reverse vaccinology is an approach used to analyse the genome of a pathogen in order to explore the complete repertoire of antigenic candidates [67, 68]. The selected candidates are then expressed recombinantly and immunogenicity is then evaluated *in vivo* [68]. The search for candidate antigens is initiated by screening for coding regions and open reading frames (ORFs), followed by homology searches using the potential antigens against known and characterised proteins. Finally, the selected potential antigens are subjected to structural and functional genomic analysis to identify their cellular location, and the candidate vaccine antigens are recombinantly expressed to examine their immunogenicity [69-72].

1.6.4 **DNA vaccines**

DNA vaccines use a bacterial plasmid encoding the antigen protein and equipped with a potent eukaryotic promoter to induce protein expression *in situ* [73]. The vaccine can be administrated via intramuscular, subcutaneous or mucosal routes, depending on which immune cell is to be stimulated [74]. The DNA constructs enter the cell nucleus and the antigenic proteins are expressed by host cell machinery, and the proteins are recognised by the host immune system and elicit immune responses [75, 76].
One key attraction to DNA vaccines is the potential to deliver antigens to both major histocompatibility complex (MHC) class I and class II pathways. In particular, the MHC class I pathway, which is responsible for the generation of cytotoxic T-lymphocyte immune responses (CTLs) is often unachievable by traditional inactivated and subunit vaccines [75, 77].

Currently, there are no DNA-based vaccines licensed for human use, but four veterinary products are available. These include West Nile virus vaccine for horses [78], hematopoietic necrosis virus vaccine for fish [79], a melanoma cancer vaccine for canines [80], and a growth hormone enhancing product for swine [81].

### 1.6.5 RNA vaccines

The concept of the RNA vaccine was first demonstrated by Wolff et al., 1990 [82], when mice immunised intramuscularly with messenger RNA (mRNA) vectors encoded with reporter proteins, demonstrated that *in vivo* protein expression from “foreign” RNA molecules was achievable [82]. Thereafter, Martinon et al., 1993 [83], described the induction of virus-specific CTLs immune responses *in vivo* in mice, by immunisation of liposome-entrapped mRNA encoding the influenza virus nucleoprotein (NP) [83]. One superior fact about the RNA vaccine is that a significant portion of the host innate immune response is responsible for sensing and counteracting viral particles with the RNA genome. Thus, certain forms of RNA molecules can stimulate potent innate immunity, which plays an important role in the subsequent generation of effective adaptive immune responses [76, 84, 85].
1.6.6 Vector vaccines

1.6.6.1 Viral vectors

The concept of using virus as vaccine vectors involves a highly-attenuated recombinant viral pathogen which infect host cells and hijacks the cellular machinery to express both viral proteins and vaccine antigens, subsequently inducing an immune response [86, 87].

Viral vector vaccines are known to elicit strong humoral immune responses, and also have the potential to activate CTLs through MHC class I presentation [86]. In addition, CTLs are known to detect and target strain-independent highly conserved antigenic epitopes, and such epitopes are often inaccessible to antibodies which are restricted by their functionality and position [86]. One such example was demonstrated by Brewoo et al., 2013 [88], where the influenza structural protein nucleoprotein (NP), derived from influenza H1N1 (A/influenza/Puerto Rico/8/34), was inserted into modified recombinant vaccinia virus Ankara (MVA). Immunised mice showed complete protection against lethal challenges from homologous H1N1 (A/Norway/3487-2/09), and heterologous H5N1 (A/Vietnam/1203/04), and partial protection against seasonal H3N2 (A/Aichi/68) [88]. The vaccine did not only induce significant levels of neutralising antibody responses, but also elicited IFN-γ secreting CD8+ and CD4+ T-lymphocytes and IL-2 secreting CD4+ T-lymphocytes [88]. This example provides a proof-of-concept that structural and highly conserved pathogenic epitopes can be carried by viral vectors and induce protective immunity.

1.6.6.2 Bacterial vectors

The use of attenuated bacteria to express heterologous antigens and serve as vaccine carriers has been extensively studied over the last 30 years [89]. Such technology provides versatile applications in vaccine development, and has been used to elicit immune responses
against bacterial, viral, protozoan and metazoan pathogens in animal models and clinical studies [89, 90]. Bacterial vaccine vectors possess many advantages: 1) they are easy and inexpensive to manufacture with flexible scalability; 2) there are multiple vaccination routes available, especially oral-mucosal routes [89, 91]; 3) enteric bacterial vectors have tropism towards lymphoid antigen presenting cells (e.g. dendritic cells and macrophage) in the intestinal mucosal tract, which is a unique asset for developing mucosal vaccines [89, 92-96]; and 4) they are known to elicit potent innate and adaptive immune responses against *Salmonella* and heterologous antigens [97, 98].

A variety of heterologous antigens expressed in bacterial vectors have been shown to confer protection against disease in animal models, but suboptimal results observed in humans and primates have stalled the development of such vaccine strategies [90, 99]. Currently, there are no licensed bacterial vaccine vectors, but substantial evidence suggest that recombinant *Listeria monocytogenes, Salmonella* and *Shigella* vaccine carriers are competent to elicit both humoral and cell-mediated immunity against a heterologous antigen [89].

Intracellular pathogens such as *Salmonella* spp. are known to establish infection via the intestinal tract, therefore oral administration can easily be achieved. Furthermore, studies have shown their ability to present homologous and heterologous antigens to both endogenous and exogenous antigen-presenting pathways, eliciting CD4$^+$ and CD8$^+$ T-lymphocyte activities, and to stimulate humoral immune responses to induce serum and secretory IgA antibodies [100-107]. Nevertheless, the concerns of reversion to pathogenic wild-type and limited immunogenicity due to pre-existing immunity against vaccine vectors, must be properly addressed for the development of such vaccine systems [2, 89].
1.7  *Salmonella*-vectored vaccines

*Salmonella* are Gram-negative bacilli, facultative anaerobic, motile, non-lactose fermenting, belonging to the *Enterobacteriaceae* family. *Salmonella* infect humans and animals generally by the oral-faecal route [108].

1.7.1  Pioneer study of *Salmonella*-vectored vaccines

The use of attenuated *Salmonella* as a carrier for heterologous protein via oral delivery has been well documented over the last few decades. In the early 1980’s, Formal *et al.*, 1981 [109] conducted a pioneering study into heterologous antigen presentation using *Salmonella* species as a vaccine carrier, where the first existing attenuated typhoid vaccine *Salmonella enterica* serovar Typhi Ty21a was used to present the form I antigen from *Shigella sonnei*. This vaccine induced protection against infection from both *S. enterica* and *S. sonnei* in immunised mice. Thereafter, a vast variety of recombinant attenuated *Salmonella* strains have been generated to target various infectious and non-infectious diseases [110].

1.7.2  *Salmonella* pathogenesis and immune responses

*Salmonella* is favorable over other vaccine carriers, based on the fact that upon *Salmonella* infection, both *Salmonella* and heterologous antigens are delivered into APCs such as DCs and macrophages - the natural target cells of *Salmonella* [111]. Furthermore, *Salmonella* pathogen-associated molecular patterns (PAMPs) elicit potent innate immune responses during infection [16], which stimulate the maturation of these APCs and migration to secondary lymph nodes, leading to the initiation of adaptive immune responses [112]. Therefore, *Salmonella* self-derived PAMPs serve as vaccine adjuvants which amplify the immune response against the heterologous antigen. *Salmonella*-based vaccine carriers also promise flexibility and versatility in regards to directing appropriate immunological reactions.
including CD4$^+$ T-lymphocytes, CD8$^+$ T-lymphocytes and regulatory T-lymphocytes, which were shown to be elicited during *Salmonella* infection, and confer protective immunity against different diseases [113-118].

Attenuated *Salmonella* has also become an attractive vector to deliver modern DNA vaccines, where the heterologous genes are not expressed, but carried by the vector. This is because *Salmonella* has the unique ability to transfer expression plasmids to host cells, even if the bacteria are unable to escape the phagocytic vacuole [119, 120].

### 1.7.3 Role of antigen presenting cells in *Salmonella* immunity

As a facultative intracellular pathogen, T-lymphocytes are vital components of the specific immune response to *Salmonella*. The bacterial specific CD4$^+$ and CD8$^+$ T-lymphocytes are essential for complete protection against *S. Typhimurium* [121]. Macrophages and immature DCs also play an important role in the elicitation of both innate and adaptive immune responses to the infection. These cells capture and degrade bacteria in a defined proteolytic pathway, and processed bacterial antigens are displayed on the APC for antigen recognition [122].

The MHC class I and the class II molecules expressed on APC surface present antigenic peptides that are accessible to CD8$^+$ cytotoxic T-lymphocyte and CD4$^+$ helper T-lymphocyte [123]. The MHC class I molecules containing antigenic fragments are transported to the endoplasmic reticulum before being transferred to the MHC molecule, and then the antigen fragment is presented via MHC molecule for recognition by CTL [123]. For MHC class II presentation, after endocytosis and fusion with the lysosome, degraded protein fragments enter the MHC class II molecule pathway [124], which suggested that the major phagocytic cells, neutrophils, macrophages and DCs all play a role in the induction of *S. Typhimurium*
specific T-lymphocytes responses. Nevertheless, DCs are believed to have a superior ability to stimulate naïve T-lymphocyte in comparison to macrophages.

1.7.4 Attenuation for a *Salmonella*-vectored vaccine

In the past, attenuation of bacteria has been achieved by chemical mutagenesis, for example *Salmonella* Typhi Ty21a [125]. However, most of the attenuated *Salmonella* strains currently used for experimental or clinical studies are auxotrophic strains which are generated via the deletion or mutation of essential genes that are accountable for biosynthesis of metabolically essential elements such as aromatic amino acids (aro), guanidine (gua) or purine (pur) [99]. These mutations attenuate the strain whilst preserving its immunogenicity. Such *Salmonella* mutants have shown promising results in animal models. For example, in guinea pigs, the orally administered *Salmonella* Typhimurium *aroA* mutant expressing chromosomally integrated *Mycobacterium tuberculosis* fusion antigen Ag85B-ESAT6 followed by boosting with a dose of purified Ag85B-ESAT6, successfully reduced the level of *M. tuberculosis* in the lung and spleen to the same extent as the BCG vaccine [126]. Another study demonstrated that the passenger antigen delivered could be detected in Peyer’s patches and the spleen several days after immunisation with the attenuated *Salmonella*, even if the expression plasmid was lost soon after administration [119]. Darji *et al.*, 2000 [120] demonstrated that even though plasmid loss took place, mice that were immunised four times with attenuated *Salmonella* encoded virulence factors of *L. monocytogenes* through oral administration were protected from a lethal challenge with *L. monocytogenes*.

1.7.5 Genetic stability and protein expression of heterologous genes

1.7.5.1 Chromosomal integration

Chromosomal integration of a cassette encoding the heterologous antigen gene allows maximum genetic stability, as chromosomal DNA rarely undergoes mutation or deletion.
However, chromosomal integration usually results in a single copy of heterologous antigen per bacterium, which is a challenge that has to be resolved to ensure sufficient antigen is expressed to confer protective immunity [128]. Consequently, the selection of a potent and inducible promoter is therefore important.

**1.7.5.2 Plasmid-based expression systems**

Plasmid-based heterologous antigen expression on the other hand, is hindered by the metabolic burden associated with plasmid replication, which can over-attenuate the carrier or lead to spontaneous loss of plasmid [129]. There are a wide variety of strategies being investigated to improve plasmid retention. One of the most successful strategies for the prevention of plasmid loss is known as the conditional lethal system. This system maintains the plasmid-bearing bacterial population by encoding replication-essential proteins in the plasmid, so that plasmid-less daughter cells are unable to survive without the plasmids [130, 131]. In one such approach, the *asd* gene which encodes aspartate β-semialdehyde dehydrogenase was used. It is an important enzyme for not only amino acid synthesis, but also for cell wall synthesis [131]. Consequently, harbouring an *asd*-encoding plasmid is essential for survival, unless the bacteria carry the chromosome-encoding *asd* gene. This particular system has been successfully demonstrated in a live attenuated *S. Typhimurium* to carry a variety of heterologous antigens including a viral peptide from HBV [132], and F1-Ag and V-Ag antigens derived from *Yersinia pestis* [133]. The *S. Typhimurium* vaccine induced potent humoral immune responses, including serum IgG and secretory IgA in mucosally immunised mice.

Another important aspect of using a vaccine carrier to deliver heterologous antigen is how the protein expression is controlled and compartmentalised. Regulating the level and location of heterologous antigen expression can have a significant impact on the
immunogenicity of a vaccine carrier. Incorporating an appropriate expression promoter is the key to guarantee the desired level and timing of antigen delivery, and to confer the optimal immune responses [134]. Early studies conducted by Hohmann et al., 1995 [134] demonstrated that an antigenic protein expressed from a constitutive promoter encoded in S. Typhimurium chromosome was incapable of inducing a protective antigen-specific immune response. In contrast, in vivo-inducible promoters such as $P_{pagC}$, which only induces antigen expression after the bacterial cell has been phagocytosed by macrophages, have induced a strong serum IgG response against the same antigen [134]. It was suggested that although the constitutive promoter confers high levels of heterologous antigen expression, there is an overall increase in metabolic burden to the vaccine carrier, which compromises immunogenicity [135].

### 1.7.6 Heterologous antigen presentation

It is known that the nature of antigen toxicity within a vaccine carrier could hinder colonisation and reduce overall antigen delivery. Protein folding is another essential element for inducing protective humoral responses against conformational epitopes. Several strategies have been developed to overcome these hurdles, which include the export of the antigen to the extracellular space and direct surface display [136, 137]. Kang and Curtiss, 2003 [138] demonstrated that in orally immunised mice, the antigen-specific humoral response of an attenuated S. Typhymurium carrying PspA derived from S. pneumonia surface protein was enhanced 10,000-fold by incorporating the secretion signal from β-lactamase compared with the unfused PspA construct. Surface display is an attractive means to present heterologous antigen to the host immune system because of the potential to elicit potent humoral immunity. Currently, heterologous genes can be fused into a gene encoding bacterial outer membrane proteins such as OmpA [139], LamB [140] or flagellin [141]. The fusion protein that contains the heterologous antigen is then presented on the bacterial
outer membrane for immunological stimulation. Another approach for surface display is to utilise the bacterial autotransporter (AT) system. The ATs are the most abundant proteins encoding many virulence factors of Gram-negative bacteria, and are responsible for exporting the N-terminal domain of the fusion protein to the bacterial outer membrane [141].

1.7.7 Immune responses against heterologous antigen

*Salmonella* as a vector vaccine carrying heterologous antigen can in theory induce both local and systemic immune responses. The understanding of *S. Typhimurium* pathogenesis and the cross-interaction between the host immune system and associated virulence factors allowed the generation of live-attenuated *Salmonella* vaccines and their application for the delivery of heterologous vaccine antigen [3].

1.7.7.1 Cell-mediated immune responses

It has been demonstrated that *Salmonella* expressed heterologous antigens can elicit Th1, Th2 and CTL responses [142]. However, the level of immune stimulation may vary depending on several factors such as the types of strains, attenuation and antigenic protein expressed [143]. The activation of a Th1 response is characterised by the up-regulation of IFN-γ; whereas the induction of a Th2 response is characterised by IL-4 [138]. Some studies also demonstrated the activation of CTL responses to the passenger antigens [144-146]

1.7.7.2 Humoral immune responses

Some studies have demonstrated that protective humoral responses against a heterologous antigen can be elicited by *Salmonella*-vectored vaccine carrier, which include bacterial, viral and parasitic antigens [147-153]. The induction of IgA antibody responses against
heterologous antigen in the intestinal tract, saliva and lung have been reported in some studies using oral administration [154-156]. The elicitation of serum IgG responses against heterologous antigen has also been demonstrated when the vaccine was orally administered [156-158].

Furthermore, the induction of both Th$_1$ and Th$_2$ responses against heterologous antigen has been observed by the presence of characteristic IgG1 and IgG2 serum antibodies, as IgG1 is an indicative of a Th$_2$ response and IgG2 is indicative of a Th$_1$ response [154, 155, 157].

### 1.7.8 Application to livestock

There have been studies demonstrating the use of *Salmonella* vaccine carriers carrying a heterologous antigen for application in livestock. Stokes *et al.*, 2007 [159] demonstrated the use of a vector expressing a *Bacillus anthracis* protective antigen which conferred protection against aerosolised *B. anthracis* spores in orally immunised mice, and had previously shown to induce protection against spore challenge through subcutaneous delivery [160].

### 1.8 Veterinary vaccines

The objectives for the development of successful veterinary vaccines can be very different from those for human vaccines, depending on the target host. Vaccines for companion animals generally have similar objectives as for human vaccines, as the health and welfare of individual animals is the main consideration for vaccine development. In livestock animals on the other hand, the cost-benefit of food production and improved overall production is the bottom line that drives vaccine development [51, 161]. It is also driven by the requirement for the protection of livestock against zoonotic pathogens, and consumers against food-borne pathogens [51]. Vaccine development for wildlife is generally restricted to the pathogens that are transmittable to humans, such as the sporadic outbreaks of highly
pathogenic avian influenza infections in Southeast Asia, and increasing interest for the development of such a vaccine has been seen in recent years [51].

In terms of economic considerations of animal vaccine development, the potential returns are much less compared to human vaccines. This is mainly due to a low sale-price or fewer market requirements, hence leading to lower investment in the research and development of animal vaccines [161]. Nevertheless, the regulations for approval of animal vaccines are usually less stringent than for human vaccines, resulting in faster market launch and investment returns, which is advantageous over the development of human vaccines [51].

Conventional veterinary vaccines are usually based on live-attenuated or inactivated pathogens, for example, the inactivated equine influenza virus vaccine [55]. Nowadays, molecular techniques are increasingly employed in the development of second generation veterinary vaccines, such as the DNA vaccine against infectious hematopoietic necrosis virus for salmon [79], and the fowl pox viral vectored vaccine against Newcastle disease virus for poultry [162].

1.9 Influenza virus

Influenza remains as one of the most important diseases in humans and animals, as the virus continuously undergoes antigenic changes and has a broad range of animal reservoirs [163]. There are three types of influenza virus: A, B and C, with the classification based on the serological reactivity of internal proteins. Influenza type B and type C are mostly restricted to humans and occasionally isolated from other mammalian hosts. Type A influenza virus on the other hand, causes disease in a wide variety of avian and mammalian species [164, 165]. As type B and type C influenza virus result in minor illness, they are not of much concern, whereas type A influenza virus has the potential to evade the human immune system and
result in widespread infection [166]. This review will explore the human and socioeconomic impacts caused by type A influenza virus infections, the viral pathogenesis and the current and potential control measures.

1.9.1 Influenza in the poultry industry

In 1878, a mysterious contagious disease in poultry associated with a high mortality rate was described in Northern Italy. The disease was initially termed “fowl plague” and was confused with the acute septicemic form of fowl cholera [167]. The disease subsequently spread along with poultry merchants to Eastern Austria, Germany, and later Belgium and France, eventually becoming endemic throughout Central Europe and Italy [168]. Decades after the first description of fowl plague, the causative agent was identified as a virus in 1901 and further determined to be a type A influenza virus in 1955. The term “fowl plague” was finally replaced by the more appropriate term “highly pathogenic avian influenza” at the First International Symposium on Avian Influenza in 1981 [167].

Type A influenza virus is sub-typed according to serological analysis, to two surface-glycoproteins known as hemagglutinin (HA) and neuraminidase (NA). To date, 17 HA and 10 NA subtypes have been identified [169]. These avian influenza viruses (AIV) are further classified according to their disease manifestations, where low pathogenic AIV (LPAIV) causes asymptomatic to mild infections, and highly pathogenic AIV (HPAIV) results in up to 100% morbidity and mortality [169-171]. Until now, there are only some strains of H7 or H5 subtypes which meet the defined criteria of being highly pathogenic, and they are believed to have evolved from low pathogenic precursors [167].

AIV have been isolated from a variety of animal species including humans, pigs, horses, mink, felids, marine mammals, domestic birds and free-flying wild birds [172]. As summarised by
Olsen et al., 2006 [172], LPAIV have been isolated from at least 105 wild birds species of 26 different families, and it is well-documented that these LPAIV from wild birds are occasionally spread along their migratory route, to the major AIV reservoirs “domestic birds” (i.e. duck, geese, terns and wader), and cause sporadic LPAIV or HPAIV outbreaks [172]. The first serologically confirmed HPAIV outbreak in poultry was in a small farm in Scotland in 1959 [173], thereafter, in excess of 28 separate HPAIV outbreaks were documented, resulting in significant economic losses [167, 174]. For instance, the H5N2 HPAIV outbreak in Pennsylvania in 1983 initiated with a low pathogenic H5N2 virus, where acute respiratory disease with increased mortality (0% to 2.7%), and decreased egg production (4% to 43%), was diagnosed in an egg-laying flock. The disease was then spread between flocks, and an estimated 100 flocks became infected between April and October of 1983 [175]. Later in the year, signs of virus mutation were seen in a layer flock, where the chickens exhibited significantly decreased food and water consumption, ceased egg production, and displayed severe comb lesions. Mortality rate increased from 50% to 89% in a 10-day observation time (Figure 1.2) [175]. Signs of neurological symptoms such as tremors were also observed, which is usually absent in LPAIV infections [175]. The virus was later isolated and identified as a highly pathogenic variant of the epidemic H5N2 LPAIV, which had begun earlier that year [175, 176]. The outbreak was eventually controlled by the mass slaughter of more than 17 million birds with direct and indirect costs of more than USD$312 million [167]. Of particular interest was that it was the first documented change of an LPAIV to an HPAIV during a field outbreak, which emphasises the fast-changing nature of type A influenza virus [175].
A) Extreme mortality in broiler chickens during the H5N2 HPAIV outbreak in Pennsylvania in 1983.

B) A layer chicken naturally infected with H5N2 HPAIV developed severe necrosis and ruptured vesicles in the comb caused by the infection. Images obtained from [175].
Except for the 1983 Pennsylvania outbreak, most of the other HPAIV outbreaks were limited to the initially infected farms with little or no spread, but occasional widespread outbreaks were documented, resulting in massive economic losses (Table 1.2).

The HPAIV H5N1 outbreak is shown in (Table 1.2) has received the most attention in recent years, mainly due to its far-reaching adverse effects in domestic birds, public health, and also poultry industries on a global scale [177]. The virus first surfaced in Hong Kong in the late 1990s and has spread across Asia, Europe and Africa in recent years [178]. It was first isolated from sick geese from a flock in the Guangdong Province of China in 1996, and the outbreak resulted in the closing and depopulation of all live-bird markets, with 18 human cases reported by the end of 1997 [177-179]. Since then, sporadic outbreaks of HPAIV H5N1 have been reported in Hong Kong, China and other parts of Southeast Asia in poultry and wild-birds, and human cases related to HPAIV H5N1 resurfaced in Vietnam in 2003 [177]. The infected host species of this HPAIV H5N1 has since expanded beyond humans, poultry and wild-birds, to infect canines, felines, swine and mustelidae, further increasing the possibility of virus-human contact [177].
Table 1.2: Significant HPAIV outbreaks since 1959

<table>
<thead>
<tr>
<th>Country</th>
<th>Year</th>
<th>Subtype</th>
<th>Species affected</th>
<th>Approximate number of bird slaughtered</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pennsylvania USA</td>
<td>1983</td>
<td>H5N2</td>
<td>chicken, turkey</td>
<td>17 million chickens</td>
<td>[167, 175, 176]</td>
</tr>
<tr>
<td>Mexico</td>
<td>1994</td>
<td>H5N2</td>
<td>chicken</td>
<td>&gt;millions</td>
<td>[180, 181]</td>
</tr>
<tr>
<td>Pakistan</td>
<td>1994</td>
<td>H7N3</td>
<td>chicken</td>
<td>&gt;6,000,00</td>
<td>[182]</td>
</tr>
<tr>
<td>Italy</td>
<td>1999</td>
<td>H5N2</td>
<td>turkey</td>
<td>14 million chickens, turkeys, guinea fowl, quails, ducks, pheasants, ostriches</td>
<td>[183-185]</td>
</tr>
<tr>
<td>Chile</td>
<td>2002</td>
<td>H7N3</td>
<td>chicken</td>
<td>2 millions</td>
<td>[186, 187]</td>
</tr>
<tr>
<td>Netherland</td>
<td>2003</td>
<td>H7N7</td>
<td>chicken</td>
<td>&gt;34 million</td>
<td>[188-190]</td>
</tr>
<tr>
<td>Canada</td>
<td>2004</td>
<td>H7N3</td>
<td>chicken</td>
<td>16 million</td>
<td>[191, 192]</td>
</tr>
<tr>
<td>Asia, Europe and Africa</td>
<td>2003- present</td>
<td>H5N1</td>
<td>chicken, duck</td>
<td>&gt;100 millions</td>
<td>[177]</td>
</tr>
</tbody>
</table>
The sporadic outbreaks of LPAIV remain underappreciated and should not be neglected, such as the LPAIV H9N2 which caused sporadic outbreaks in Europe, Asia, Africa, the Middle East and the U.S., and is now considered enzootic throughout Asia [174]. It is reported that LPAIV H9N2 sporadically causes human infections with flu-like symptoms, which is indistinguishable from the seasonal human influenza strains H1N1 and H3N2 [193, 194]. Although there is no evidence to strongly indicate human-to-human transmission, given the predicted low affinity of LPAIV H9N2 for human influenza receptors, the recurring transmission into the human population increases the possibility of influenza viral re-assortment and subsequent ability for human-to-human transmission [182, 195-197].

1.9.2 Influenza infection in humans

In the last 100 years, there were five unquestioned human influenza pandemics occurred in 1918 (H1N1), 1957 (H2N2), 1968 (H3N2), 1977 (H1N1) and 2009 (H1N1) [198]. Among them, the “Spanish flu” is the worst known pandemic in recorded history, and claimed an estimated 50 million human deaths or more, worldwide (Figure 1.3) [199, 200].

The 1918 H1N1 influenza pandemic exhibited several unique clinical and epidemiological characteristics, as the clinical course was usually self-limiting, but a high percentage of infected victims developed severe pneumonic complications [201].
Figure 1.3: The H1N1 pandemic outbreak “Spanish flu” in 1918.

Emergency military hospital during the influenza pandemic in the United States in 1918.

Image obtained from [166].
Furthermore, epidemiological features of the 1918 pandemic were unprecedented, where the virus struck in three separate waves between spring of 1918 and winter of 1919, and an unusual “W-shaped”, age-specific mortality curve characterised by an unexplained peak death in healthy young adults between 20 and 40 years of age [201-203]. A recent phylogenetic study by Worobey et al., 2013 [204] has suggested that the age-specific mortality in 1918 may have been due primarily to their childhood exposure to a doubly heterosubtypic H3N8 virus, which was estimated to have circulated between 1889 and 1900; whereas, all other age groups were probably partially protected by childhood exposure to H1 or H1-related antigens.

The subsequent influenza pandemic emerged in 1957-1958 and the causative virus type A influenza (H2N2), was a lineal descendent of the 1918 H1N1 “killer” strain that had acquired three novel genes from avian-like subtypes. The original surface-glycoproteins were replaced with H2 and N2, and a gene encoding the PB1 polymerase was also replaced with an avian-like gene segment [205, 206]. It is still uncertain in which intermediate host the viral re-assortment took place, or how long the re-assorted virus took to adapt into an efficient human-to-human transmission pandemic [201]. The pandemic eventually killed an estimated two million people worldwide [207]. The virus then became seasonal endemic and sporadic in 1959, and vanished within 11 years [201].

Also known as the “Hong Kong flu”, type A influenza once again gained novel avian genes by re-assortment with the previously circulating virus, which lead to the pandemic outbreak in 1968 [200]. The causative agent responsible for the 1968 pandemic was a type A influenza H3N2, where the previous H2 and PB1 genes were replaced with avian-like H3 and PB1 to create yet another descendent of the 1918 virus [200, 205, 206]. Despite the novelty of the newly re-assorted influenza H3N2 virus, the aggressiveness of the H3N2 pandemic was
modest in its mortality impact, and quickly became seasonally endemic with sporadic appearance, and remains circulating worldwide to date [201].

The latest influenza pandemic emerged in Mexico in 2009. The virus was known as the type A influenza H1N1 “Swine flu”, where it was a genetically re-assorted strain between two pre-existing swine lineage viruses – a North American H1N2 and a Eurasian H1N1 [208, 209]. The outbreak initiated in Mexico, where severe pneumonias were reported from infected individuals. Nevertheless, the pathogenicity of “Swine flu” virus was mostly self-limiting and clinically similar to the seasonal influenza virus in other countries [210]. As of 19th March 2010, WHO has reported at least 16,813 reported human casualties from millions of infected individuals [201].

Unfortunately, despite progressive accumulation of scientific knowledge of influenza viruses, it is still impossible to accurately predict whether or not a pandemic is imminent, when or where it may emerge, and what sort of subtype and global impact it may have, as evidenced by the completely unexpected outbreak of the 2009 H1N1 virus [211, 212]. One influenza subtype of concern for a possible future pandemic is the HPAIV H5N1, as it has the potential to have an estimated 60% mortality rate, and sporadic reappearances in avian species spill over into the human population [213, 214]. Moreover, although the predominant human cases are restricted to poultry-to-human transmission, given the fast mutating nature of type A influenza virus, it is possible that the virus will adapt to human-to-human transmission and result in another devastating influenza pandemic [214].
1.9.3 The virus

1.9.3.1 Influenza viral structure

The type A influenza virus is an RNA virus which belongs to the Orthomyxoviridae family. Its genome consists of eight single-stranded negative-polarity RNA segments that encode a total of eleven proteins (HA, NA, NP, M1, M2, NS1, NEP, PA, PB1, PB1-F2, and PB2), and virus subtype is based on 17 different HA and 10 different NA envelope surface-glycoproteins [169, 215, 216]. The general structure of an infective influenza virion is made up of a lipid bilayer envelope derived from the infected host cell, two surface-glycoproteins, an ion channel protein and the RNA genome (Figure 1.4) [216].

The highly antigenic surface-glycoprotein HA is responsible for viral entry by binding with the host cellular receptor sialic acid [217]; whereas the NA is responsible for preventing viral aggregation in the infected host cell and plays an important role in allowing the ready dispersal of newly propagated virus [163]. The M1 protein located in the viral lipid envelope facilitates viral assembly, and the M2 protein serves as an ion gateway. The M2 protein is situated between the inner and outer lipid bilayer and regulates pH to facilitate HA synthesis [163, 218]. The non-structural protein (NS1) is known to induce apoptosis, proteosomal degradation and RNA editing which is believed to play a vital role in influenza pathogenesis [219, 220], and the RNA segment-coating nucleoprotein (NP) and polymerase PB1, PB2 and PA assemble the transcriptional complex essential for viral replication [221].
Figure 1.4: A schematic diagram of an influenza virion.

(Image obtained from [222])
1.9.3.2 Antigenic “shift” and “drift”

As a successful pathogen, type A influenza virus relies on two evolutionary mechanisms, “antigenic shift” and “antigenic drift” [223]. Type A influenza viruses gain entrance to host cells via HA binding to “sialic acid” receptors on the cell surface, and the binding affinity of HA to sialic acid receptor is thought to play an important role for the host specificity of several type A influenza subtypes [224, 225]. The high-degree of antigenic variation of influenza HA leads to repeat epidemics and occasional pandemics. Expeditious HA mutation is facilitated via inaccurate RNA-dependent RNA polymerase during viral genesis. It is estimated that each replication cycle generates errors at a rate of 1 in $10^4$ nucleotide bases, which is approximately $10^5$-fold more errors compared to DNA-dependent DNA polymerase replication [165, 223]. These point mutations result in amino acid substitutions in the influenza proteome and those which occur within the surface-glycoproteins allow the virus to evade the host immune system despite the presence of pre-existing humoral immunity. This event is termed “antigenic drift”. It has been reported that a minimum of four amino acid substitutions distributed on minimum of two or more antigenic sites of the HA are sufficient to allow re-infection [226, 227].

The segmented RNA genome of type A influenza virus also contributes to another antigenic variation known as “antigenic shift” or viral re-assortment [228]. The re-assortment could happen when two or more different influenza subtypes from different origins co-infect a single animal. Given the presence of a vast reservoir of type A influenza viruses in avian species thus raises the variety of viral subtypes available for re-assortment [163]. Such events enable the virus to evolve and become antigenically novel. The virus then has the potential to become a pandemic viral strain which can evade the host immune system and cause widespread infection, such as the 2009 H1N1 swine-origin influenza virus [223].
It has been documented that, human influenza has preferential tropism of binding with sialic acid linked to galactose by α-2, 6 linkages, whereas avian viruses of this type prefer to bind to α-2,3 linkages. Given that many animals, including swine, chickens and humans have both types of linkages on their epithelial cells, animals co-infected with different subtypes of influenza virus may serve as “mixing vessels” for the generation of novel virus [216]. Moreover, the “wet market” of Southeast Asia, where humans, swine, poultry and occasionally other animals are in close proximity further increases the risks to public health [229-231].

1.9.3.3 The role of Hemagglutinin in influenza pathogenesis

Influenza HA belongs to the type-1 membrane-glycoprotein family and has three major activities in viral pathogenesis; 1) the HA specifically binds to cellular receptors containing sialic acid on target cells to initiate the virus-cell interaction, 2) the HA mediates the viral entry into the cell cytoplasm by membrane fusion, and 3) the HA is the major target site for humoral immune responses [232].

To infect the host cell, the HA first binds to the host receptor and is then internalised by endocytosis. The low pH in the endosome drives conformational changes in HA which results in fusion of the viral membrane with the endosomal membrane, consequently allowing the viral genome to enter the cell (Figure 1.5) [233].
Figure 1.5: The illustration of a typical influenza viral entry.

Influenza HA1 is responsible for initial attachment to the host cell receptor containing sialic acid. Image adapted from [234].
Once the infection has been established, the immature HA is synthesised as a single polyprotein, which is a precursor of mature HA [235]. The HA precursor contains two domains, one sialic acid receptor binding domain (HA1) and the ectodomain (HA2), also known as a fusion peptide which anchors to the viral lipid bilayer [236]. The HA precursor polyprotein must be post-translationally cleaved by host proteolytic activities at a conserved arginine residue to form two domains, HA1 and HA2 which are connected by a disulfide bond [237]. This cleavage event is vital for viral infectivity, as the HA cleavage releases the hydrophobic N-terminus of HA2 which is inserted into the target membrane during fusion. The cleavage also allows the molecule to be structurally functional, and then activated by the acidic environment of the endosome to undergo the molecular rearrangements necessary for fusion [237]. The HA precursor contains monobasic cleavage sites for extracellular cleavage by trypsin-like serine proteases, which may originate from host epithelial cell proteases, host inflammatory proteases, as well as bacterial proteases [165]. This also explains why a remarkable 75% of influenza patients who developed severe pneumonia are diagnosed with bacterial co-infections, as bacterial proteases may assist the activation of functional HA [237-240].

Influenza HA is the most immunogenic component of the influenza virus, as a significant amount of humoral immune response following influenza infection in animals and humans is directed toward the HA, with a population of these antibodies capable of neutralising HA binding and/or fusion [241-245]. Early investigations have revealed that these antibodies have a negative correlation with disease manifestation in animals, thus suggesting that these antibodies are essential for the protection against influenza infection [241, 246-250]. In addition, most of the influenza virus protective antigenic sites are conformational epitopes and located predominantly on the HA1 globular head [251, 252].
1.9.4 Disease control and prevention

Despite ongoing annual vaccine programs, seasonal influenza epidemics continue to be a major cause of high mortality and morbidity. The currently employed egg-based “inactivated whole-virus vaccine” only confers short-term and highly specific humoral immunity, which is incompetent to deal with the rapidly evolving influenza virus. Most importantly, the i.m. administered vaccine is unable to elicit protective mucosal immunity at the site of viral entry [1], and manufacturing of the egg-based vaccine is both time-consuming (up to nine months) and unsuitable for egg-allergy individuals [253].

In the poultry industry, outbreaks of HPAIV such as H5N1 are responsible for significant economic losses and also pose serious threats to public health, therefore control measures not only help to limit industrial and economic losses but also reduce risks associate with human infections [254-256]. Currently, the industry relies on enhancing biosecurity measures, frequent serological surveillance, and restricting the movement of birds to control and prevent an influenza epidemic in poultry [170, 177]. However, these control measures have not been able to prevent the virus spread since they first emerged [257]. In recent years, vaccines for poultry influenza have been introduced in some developing countries to limit the socioeconomic impact of HPAIV outbreaks in poultry [169, 258]. Nevertheless, the current vaccines have not been the panacea for the prevention of avian influenza in the field, as there are several limitations and concerns associated with vaccine efficiency, including:

1) Multiple subtypes might be co-circulating in some epidemic regions [259].

2) Vaccine-induced antibodies could interfere with routine serological surveillance and differentiation of diseased birds from vaccinated birds [260].

3) Vaccine-induced immune pressure may accelerate viral antigenic drift to evade pre-existing immunity [261-263].
4) Regular “updates” of vaccine serotype are required to control the antigenic drifted variants [264, 265].

5) Vaccinated birds might still be infected but absent of clinical symptoms, thus leading to “silent” circulation of the virus [266, 267].

6) Vaccine efficacy and response could vary between poultry species, even within the same species [268-271].

7) The duration of vaccine-induced protective immunity varies [272].

8) Young birds carrying maternally acquired immunity through vaccination could influence subsequent vaccination [273, 274].

1.9.5 Avian influenza vaccines

The routinely used vaccines against avian influenza in the poultry industry are for control of LPAIV H9N2 in Asia, LPAIV H5N2 in Central America, and HPAIV H5N1 in Asia and Africa [170]. Based on experimental studies and field usage, vaccines against AIV can be classified into four technological groups: 1) inactivated whole influenza virus, 2) in vitro expression of subunit HA protein, 3) in vivo expression of HA protein, and 4) nucleic acid vaccines [170]. Although the live-attenuated avian influenza vaccine promises to induce superior protective immunity, the potential to mutate into a novel HPAIV could result in considerable economic losses and possible human infections. Thus, live-attenuated avian influenza virus is presently discouraged from vaccine use in the poultry industry [275, 276].

1.9.5.1 Inactivated whole-virus vaccines

Similar to the production of human seasonal influenza vaccines, the method of developing an inactivated whole-virus vaccine is low cost and technologically “mature”, and has been used for more than 30 years [275]. In the past, the LPAIV isolated from outbreaks and/or serological surveillance from wild and domestic birds have been the primary viral strains
used in vaccine development. Nowadays, some HPAIV have been used in inactivated influenza vaccines such as H7N3 in Pakistan and H5N1 in Indonesia [170]. Currently, the overwhelming majority of avian influenza vaccines manufactured and used in the poultry industry are oil-adjuvanted inactivated whole-virus vaccines, which administered by parenteral injection [275]. However, limited use of inactivated avian influenza vaccines has been reported due to high labour costs for parenteral administration of the vaccine, and the intricacies in differentiating “diseased” and “vaccinated” birds by routine serological surveillance in the vaccinated population [260].

1.9.5.2 DNA vaccines for influenza virus

Plasmid-based DNA vaccines expressing the avian influenza HA gene under the control of a promoter have been studied as an AIV vaccine, and results showed successful elicitation of protective immunity in chickens upon challenge with HPAIV H5 and H7 subtypes [277-280]. An alternative method to deliver DNA vaccines using a bacterial vector was evaluated by Pan et al., 2010 [281], where complete protection from intranasal HPAIV H5N1 challenge in chickens was achieved by priming with a HA DNA vaccine delivered orally in attenuated S. Typhimurium followed by boosting with a killed viral vaccine.

1.9.5.3 In vitro expression of influenza vaccine antigen

1.9.5.3.1 Recombinant influenza HA as influenza vaccine

Influenza HA is unquestionably the most immunogenic antigen within the influenza virus. Therefore, recombinantly expressed HA could be used as subunit vaccine and elicit protective immunity [228, 282]. Recombinant HA has been demonstrated to be expressed in vitro in mammalian cells, insect cells, plant cells, bacteria, viruses or yeasts [98, 283-289]. After expression, the protein is subjected to downstream process including extraction,
purification, and combining with an adjuvant. The subunit HA vaccine has a superior safety profile as no viable virus is involved in the manufacturing process, and it provides a more rapid and cost-effective production alternative [275, 289]. One such example was the use of baculovirus vectors to express HA from both H5 and H7 subtypes. Poultry immunised with purified HA were protected from H5 and H7 HPAIV challenge [284].

1.9.5.3.2 Yeast Expression System “Pichia pastoris”

In general, yeasts are an excellent platform for expressing heterologous proteins. Methylotrophic yeast such as *P. pastoris* can be easily and rapidly grown at high cell-density and no sophisticated facilities are required. *P. pastoris* also has several advantages over other eukaryotic expression systems, and the most important of all for the purpose of expressing recombinant vaccine antigen is the diverse post-translational modifications, which including glycosylation, peptide-folding, proteolytic cleavages and targeting secretion [235, 290-292]. A study conducted by Saelens et al., 1999 [235] has demonstrated that mice immunised with *P. pastoris* expressed influenza (A/Victoria/3/75 (H3N2-subtype)) HA were completely protected from homologous challenges and exhibited increased survival rate in heterologous challenges.

1.9.5.4 In vivo expression of influenza vaccine antigen

1.9.5.4.1 Current status of vector vaccines for the prevention of influenza infections in poultry

Various viral and bacterial vaccine vectors have been investigated for *in vivo* expression of the influenza HA gene for the purpose of creating an avian influenza vaccine. A variety of viral vectors have been used in vaccine development including viruses belonging to the Herpesviridae, Poxviridae, Adenoviridae and Retroviridae families [283, 287, 288, 293-296]. Among them, recombinant fowlpox virus has been the most extensively investigated viral vector for carrying HPAIV HA gene derived from either H5 or H7 avian influenza subtypes.
and fowlpox vector vaccines carrying HA derived from H5 subtypes have been commercialised for field applications in Mexico [302, 303]. However, there are a few limitations in regard to the use of recombinant fowlpox virus as an influenza vaccine, which are: 1) the vaccine must be administered parenterally, 2) it can only be used in chickens, and 3) chickens must be naïve to the fowlpox vector for the vaccination to be effective [170, 272]. The newly developed recombinant viral vector derived from the Newcastle disease virus on the other hand, can overcome the limitations associated with the inter-species barrier by administration via aerosol spray or eye drop [296, 304-306]. However, the concerns of pre-existing immunity to Newcastle disease virus could hinder vaccine efficacy.

On the other hand, recombinant bacterial vectors have been investigated to a lesser extent for the purpose of AIV vaccine development. Such a vaccine system can be achieved by incorporating genes encoding antigenic influenza proteins together with a potent promoter into either a bacterial chromosome or plasmid, and in vivo expression of immunogen after administration [127, 129, 307]. Georgio et al., 1997 [307] evaluated the use of L. monocytogenes expressing influenza nucleoprotein (NP), and showed that mice vaccinated intravenously cleared the influenza virus from the lungs faster after subsequent challenge. Moreover, Ben-Yedidia and Arnon, 2005 [308] demonstrated that both B- and T-lymphocyte epitopes of the influenza virus can be co-expressed within Salmonella flagellin, hence the recombinant flagellae served as an antigen carrier and also as an immunostimulant. Mice immunised Intranasally with recombinant Salmonella were protected against subsequent challenge.

1.9.5.4.2  *Salmonella*-vectored vaccine for the prevention of influenza virus

Currently, there is only limited investigation using *Salmonella*-vectored vaccine carriers for the purpose of AVI prevention in both the public health and veterinary sector. A table which
summarises the currently available literatures of the studies using *Salmonella*-vectored vaccines for the prevention of influenza is provided (Table 1.3).

In Table 1.3, the use of attenuated *Salmonella* as a means to deliver a DNA vaccine carrying AIV HA and boosting with an inactivated vaccine confers not only a mucosal IgA response, but also systemic protection against lethal challenge [281, 309]. A study conducted by Jazayeri *et al.*, 2012 [310], demonstrated the administration of a DNA vaccine carried by *Salmonella* enhanced the expression of cytokine secretion including IL-1β, IL-12β, IL-15 and IL-18 in chickens, and elevated virus-neutralising humoral response, and further studies are required to evaluate whether the vaccine construct protects against AIV infection.

The use of a *Salmonella*-vectored vaccine to deliver heterologous influenza vaccine antigen has been studied to a lesser extent. The current studies using influenza structural proteins as vaccine antigens have demonstrated suboptimal protection against challenges in animal models, and require multiple immunisations to confer complete protection in homologous challenges [311], or provided only limited immunogenicity when the vaccine constructs were administered alone [312]. The study conducted by Liljebjelke *et al.*, 2010 [312], using influenza HA1 as passenger antigen has demonstrated limited protection to low-dose challenges against homologous and heterologous virus, however no protection was conferred to high-dose challenges.

Although these vaccine vectors were less than ideal for field application, the outstanding safety profiles and the ability to induce antigen-specific immune responses suggested that the development of *Salmonella*-vectored influenza vaccines could be achieved in the future by better expression of heterologous antigens from the selected *Salmonella* vector.
Table 1.3: *Salmonella*-vectored vaccine carriers for the prevention of influenza infection in animal model studies

<table>
<thead>
<tr>
<th>Vaccine strain</th>
<th>Passenger protein</th>
<th>Animal host</th>
<th>Mode of antigen delivery</th>
<th>Outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S.</em> Typhymurium <em>aroA</em> SL7207</td>
<td>HPAIV H5N1 (A/Goose/Jiangsu/1/2000) HA</td>
<td>Chicken</td>
<td>DNA vaccine</td>
<td>Orally immunised chickens followed by inactivated vaccine provided complete protection against HPAIV H5N1 intranasal challenge, and significantly increased neutralising antibody levels and intestinal mucosal IgA. Elevated cell-mediated immune responses were observed.</td>
<td>[281]</td>
</tr>
<tr>
<td><em>S.</em> Typhymurium <em>aroA</em> SL7207</td>
<td>LPAIV H9N2 (A/Ck/China/N/2005) HA</td>
<td>Chicken</td>
<td>DNA vaccine</td>
<td>Orally immunised chickens followed by inactivated vaccine prevented virus shedding after intranasal challenge with the H9N2 strain. Before challenge, immunised chicken exhibited an elevated virus-neutralising antibody titre, and mucosal IgA titre.</td>
<td>[309]</td>
</tr>
<tr>
<td><em>S.</em> Typhymurium SV4089</td>
<td>HPAIV H5N1 (A/Ck/Malaysia/5858/04) HA</td>
<td>Chicken</td>
<td>DNA vaccine</td>
<td>Orally immunised chickens had enhanced IL-1β, IL-12β, IL-15 and IL-18 expression in the spleen, and elevated virus-neutralising antibody titre.</td>
<td>[310]</td>
</tr>
<tr>
<td><em>S.</em> Typhymurium SV4089</td>
<td>HPAIV H5N1 (A/Ck/Malaysia/5858/04) HA, NA and NP</td>
<td>Chicken</td>
<td>DNA vaccine</td>
<td>Virus-specific immune responses were not evaluated. The study demonstrated orally administered <em>S.</em> Typhymurium SV4089 was able to migrate through different organs of chicken, but did not cause septicaemia. Furthermore, <em>Salmonella</em>-specific O antiserum was elicited.</td>
<td>[313]</td>
</tr>
</tbody>
</table>
Table 1.3: *Salmonella*-vectored vaccine carriers for the prevention of influenza infection in animal model studies (Continued)

<table>
<thead>
<tr>
<th>Vaccine strain</th>
<th>Passenger protein</th>
<th>Animal host</th>
<th>Mode of antigen delivery</th>
<th>Outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. Typhymurium</em> SifA⁻ mutant</td>
<td>LPAIV H1N1 (A/WSN/1933) NP</td>
<td>Mouse</td>
<td>Heterologous antigen</td>
<td>Orally immunised mice followed with 3 booster immunisations were completely protected against lethal influenza virus challenge, and 66% protected from 2 booster immunisations. In addition, Th₁ immune responses were elicited against influenza NP.</td>
<td>[311]</td>
</tr>
<tr>
<td><em>S. Typhymurium</em> LB5010 (r⁻m⁺) &amp; <em>S. Typhymurium</em> BRD509</td>
<td>HPAIV H5N1 (A/whooper swan/Mongolia/3/2005) HA1</td>
<td>Chicken</td>
<td>Heterologous antigen</td>
<td>Orally immunised mice were completely protected against low-dose challenge with homologous strain, and partial protection from low-dose challenge from heterologous strain. However, vaccine vectors did not confer protection to high-dose challenge of either of these viruses.</td>
<td>[312]</td>
</tr>
<tr>
<td><em>Salmonella Enteritidis</em> strain (aroA htrA⁻)</td>
<td>Highly conserved avian influenza M2e gene, fused with immune-enhancing CD154 peptide sequence</td>
<td>Chicken</td>
<td>Heterologous fusion protein</td>
<td>The vaccine vector exhibited the ability to invade liver and spleen without noticeable adverse reactions. Orally immunised chickens elicited an M2e-specific antibody response, and offer significant protection against direct challenge with LPAIV H7N2, but not LPAIV H5N1.</td>
<td>[314]</td>
</tr>
</tbody>
</table>
1.10 Concluding remarks

Vaccine development has evolved exponentially over the last couple of centuries, from the primitive variolation techniques for smallpox prevention [8] to the latest nucleic acid vaccines [315]. There have been many successes in vaccine innovation which have improved the quality of life and reduced mortality for some diseases such as the polio vaccine and the more recent HPV vaccines [316, 317]. The quest towards an effective vaccine to induce long-lasting protection against recurrent disease such as the avian influenza virus would be of enormous value to both public health and the poultry industry. Many investigations have illustrated that employing an attenuated bacterial vector to deliver heterologous antigens can induce both innate and adapted immune responses to both the vector and the heterologous antigen [99, 119, 120, 318, 319]. Over the last few decades, recurrent and sporadic outbreaks of avian influenza virus in the poultry industry and occasional spill-over to the human population have raised the awareness of inadequate vaccine strategies in the industry. By careful selection of vaccine vectors and appropriate expression of immunogenic antigens, avian influenza could be controlled more easily and therefore minimise human spill-over infections. Vaccination would prevent potential widespread infections or pandemics, saving millions of dollars for the poultry industry and avoiding catastrophic impact to the already overstretched public health infrastructure.

1.11 Rationale of the project

In my previous study, the gene encoding HA1 derived from influenza model strain (A/Puerto Rico/8/34(H1N1)) was cloned into a yeast expression vector encoded with a methanol inducible promoter. The recombinant HA1 (rHA1) was successfully expressed from P. pastoris and secreted into culture media, and had been successfully purified using Nickel-charge immobilised-metal affinity chromatography (IMAC).
In this project, the first aim was to evaluate the immunogenicity of *P. pastoris* expressed rHA1 in a mouse trial. A parallel study was conducted by the collaborating institute - Institute of Biotechnology (IBT), Vietnam Academy of Science and Technology, where the HA1 gene derived from Influenza A virus (Hatay/2004/(H5N1)) was expressed in *P. pastoris*, and immunogenicity of the rHA1 was examined in chickens (personal communication).

*Salmonella*-vectored vaccines are still in the development phase in the field of modern medicine for both human and animal use. Such vaccines can be delivered in the same manner as a natural infection, thus the elicited immune responses mimics and targets specific points of the natural infection. In order to translate such advantages to field application, an established attenuated *Salmonella* Typhimurium vaccine (STM1) which has been licensed and is commercially available to the livestock industry to prevent Salmonellosis in poultry [320], was chosen as the carrier vector. Previous investigation indicated that STM1 has the ability to express the model heterologous antigen ovalbumin [4]. Upon immunisation in mice, the STM1 was not only taken up by the DCs but also lead to CD8\(^+\) T-lymphocyte responses specific to the dominant ovalbumin epitope –SIINFEKL, and mucosal IgA responses [3]. These promising characteristics lead to the development of a vaccine carrier using *Salmonella* STM1.

In this study, *Salmonella* STM1 was genetically engineered to carrier and express influenza HA1 *in vivo*. The HA1 was chosen as the vaccine antigen because it has a majority of the protective antigenic sites, and a portion of these HA1-specific antibodies have a negative correlation to the manifestation of disease [241-247]. Pre-existing immunity to the *Salmonella*-vectored vaccine carrier has been documented; however, the vaccine efficacy was not hindered in the case of delivering ovalbumin in mice [2, 4].
The aims of this project includes:

- To evaluate the immunogenicity of *P. pastoris* expressed rHA1 in a mouse model.
- To clone influenza HA1 gene into expression plasmids in *Salmonella* STM1, where different expression strategies were employed to display the rHA1 in various destinations to optimise protein expression and immunogenicity.
- To evaluate the immunogenicity of these *Salmonella* STM1 vaccine strains in chickens.
Chapter 2
General Materials and Methods
2.1 General procedures

All chemical reagents used were of analytical laboratory grade. All autoclavable materials including media, solutions and glassware were sterilised by autoclaving at 121°C (15 lbs/in²) for 20 minutes unless otherwise stated. All solutions and media were prepared with deionised water acquired from a Millipore Milli-Q® water system (Millipore, Australia). Glassware was washed with Pyroneg detergent (JohnsonDiversey, Pty. Ltd., Australia), and washed with a dishwashing machine (Gallay Scientific, Australia). Prior to use, glassware was rinsed twice with deionised water.

Solutions were dispensed using Eppendorf Research® adjustable pipettes (Eppendorf, Australia) which included 0.5 – 1.0 μL, 20-200 μL, 100-1000 μL digital pipettes, and Finnpipette® digital pipette range (Thermo Labsystems, USA) which included a 1-5 mL, 5-10mL digital pipette, and BioPette™ Plus Autoclavable Multichannel Pipettes (Labnet International, USA) which included 5-50 μL, and 20-200 μL multi-channel pipettes. Solution volumes of 1.5 mL or less were centrifuged with an Eppendorf microcentrifuge 5415C or 5415D. Volumes greater and up to 50 mL was centrifuged in a Beckman Allegra 21R centrifuge. Sartorius analytical top loading balance was used to weigh reagents greater less than 2 g; whereas reagents of greater than 2 g were weighed on an ISSCO model 300 top loading balance. Media containing supplements were autoclaved and cooled to 55°C before the addition of the supplements. All media was prepared using aseptic technique and stored at 4°C until use.
CHAPTER 2

2.2 General chemicals and equipment

2.2.1 Equipment

Balance:
- ISSC Model 300 (0.01-300g) - Industrial & Scientific Supply, Australia
- Satorius BP210S (0.1mg-210g) - Satorious Gottingen, Germany
- Biosafety class II cabinet (Aura 2000 M.A.C.) - BIOAIR Instrument, Italy

Centrifuges:
- Eppendorf AG 22331 bench top - Eppendorf Hamburg, Germany
- Heraeus Multifuge 1s-r Centrifuge - Thermo Electron, USA
- Beckman Allegra. 21R Centrifuge - Beckman Coulter, Australia
- Beckman Coulter Optima L100 XP Ultracentrifuge - Beckman Coulter, Australia

Dry block heater - Ratek, Australia.

Electrophoresis power supply – powerPac 300 - Biorad Laboratories, USA

Electrophoresis units – DNA:
- Sub-Cell wide mini - BioRad Laboratories, USA
- Mini-Sub® Cell GT - BioRad Laboratories, USA
- Electroporation cuvette - BioRad Laboratories, USA
- Gel Doc image system - BioRad Laboratories, USA

Electrophoresis units – Protein:
- Mini Protean® Tetra System - BioRad Laboratories, USA.
- Trans-blot electrophoretic transfer cell (mini) - Ratek, Australia.
- iBlot® Gel Transfer Device - Invitrogen, USA
- iMark™ microplate absorbance reader (96-well) - BioRad Laboratories, USA

Microscope:
- Olympus CX31 – light microscope - Olympus, Japan
- Olympus CKX41SF – inverted microscope - Olympus, Japan
- Olympus E330 – Camera - Olympus, Japan
Microplate luminometer – LUMItstar Omega

PCR machines:
- G-Storm GS1 Thermal Cycler
- GeneAmp PCR system 2400

pH meter

Platform shaker

Pulse controller & Gene pulser apparatus

Roller mixer

Sonifier – digital cell disruptor

Incubator (with orbital shaking) BL8500

Incubator (CO₂ incubator) Galaxy 1705

Transilluminator (UV)

Optima L80 XP Ultracentrifuge

Vortex mixer

Waterbath

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microplate luminometer – LUMItstar Omega</td>
<td>BMG LABTECH GmbH, Germany</td>
</tr>
<tr>
<td>PCR machines:</td>
<td></td>
</tr>
<tr>
<td>G-Storm GS1 Thermal Cycler</td>
<td>G-Storm, UK</td>
</tr>
<tr>
<td>GeneAmp PCR system 2400</td>
<td>Applied Biosystem, USA</td>
</tr>
<tr>
<td>pH meter</td>
<td>Metrohm, USA</td>
</tr>
<tr>
<td>Platform shaker</td>
<td>Ratek, Australia</td>
</tr>
<tr>
<td>Pulse controller &amp; Gene pulser apparatus</td>
<td>BioRad Laboratories, USA</td>
</tr>
<tr>
<td>Roller mixer</td>
<td>Ratek, Australia</td>
</tr>
<tr>
<td>Sonifier – digital cell disruptor</td>
<td>Branson, USA</td>
</tr>
<tr>
<td>Incubator (with orbital shaking) BL8500</td>
<td>Edwards Instrument, Australia</td>
</tr>
<tr>
<td>Incubator (CO₂ incubator) Galaxy 1705</td>
<td>New Brunswick™, USA</td>
</tr>
<tr>
<td>Transilluminator (UV)</td>
<td>Noves, Australia</td>
</tr>
<tr>
<td>Optima L80 XP Ultracentrifuge</td>
<td>Beckman Coulter, Australia</td>
</tr>
<tr>
<td>Vortex mixer</td>
<td>Ratek, Australia</td>
</tr>
<tr>
<td>Waterbath</td>
<td>Thermoline Scientific, Australia</td>
</tr>
</tbody>
</table>

### 2.2.2 Consumables

Centricon® centrifugal devices | Millipore, Australia |

Centrifuge tubes:
- Eppendorf tubes
- 10 mL tubes
- 15 mL tubes
- 50 mL tubes
- Ultra-Clear™ thinwall tube

<table>
<thead>
<tr>
<th>Tube</th>
<th>Supplier/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eppendorf tubes</td>
<td>Sarstedt, Australin</td>
</tr>
<tr>
<td>10 mL tubes</td>
<td>Sarstedt, Australia</td>
</tr>
<tr>
<td>15 mL tubes</td>
<td>Corning, USA</td>
</tr>
<tr>
<td>50 mL tubes</td>
<td>Corning, USA</td>
</tr>
<tr>
<td>Ultra-Clear™ thinwall tube</td>
<td>Beckman Coulter, Australia</td>
</tr>
</tbody>
</table>

Cover slips | Mediglass, Australia |

Cryovials (1.8 mL) | Nalgene Company, USA |

Filter (0.2 μm and 0.45 μm) | Sarstedt, Germany |

iBlot® Transfer Stack, nitrocellulose, regular | Invitrogen, USA |

iBlot® Transfer Stack, nitrocellulose, mini | Invitrogen, USA |

Microscope slide | LOMB Scientific Co., Australia |
2.2.3 Commercial kit for nucleic acids manipulation

Tetro cDNA Synthesis Kit
   Bioline Pty. Ltd., Australia

TOPO® TA Cloning Kit
   Invitrogen, USA

QIAprep® spin miniprep kit
   QIAGEN, Australia.

QIAquick gel extraction kit
   QIAGEN, Australia.

2.2.4 Animals used in this study

BALB/c mice (female)
   Animal Resource Centre, Canningvale, Western Australia.

Brioler Chickens (mix-gender)
   Inghams Enterprises, Packenham, Victoria

2.2.5 Materials

2.2.5.1 General media

**Brain Heart Infusion (BHI) broth:** 3.7% (w/v), prepared according to manufacturers specification (Oxoid, UK).

**Luria-Bertani (LB) broth:** 0.5% (w/v) yeast extract (Oxoid, UK), 1% tryptone (Oxoid, UK), 0.5% sodium choride (BDH Chemicals, UK).

**Luria-Bertani (LB) agar:** 0.5% (w/v) yeast extract (Oxoid, UK), 1% tryptone (Oxoid, UK), 0.5%
sodium chloride (BDH Chemicals, UK), 1.2% (w/v) bacteriological agar (Oxoid, UK).

**Nutrient Broth (NB):** 1.3% (w/v), followed manufacturer instruction (Oxoid, UK).

**Mueller-Hinton (MH) Broth:** 2.1% (w/v), followed manufacturer instruction (Oxoid, UK).

**MacConkey agar (MCA):** 5.2% (w/v), followed manufacturer instruction (Oxoid, UK).

**S.O.C. medium:** 0.5% (w/v) yeast extract (Oxoid, UK), 2% tryptone (w/v) (Oxoid, UK), 10 mM sodium chloride (BDH Chemicals, UK), 2.5 mM potassium chloride (BDH Chemicals, UK), 10 mM magnesium chloride (BDH Chemicals, UK), 10 mM magnesium sulfate (BDH Chemicals, UK). Media was autoclaved and stored at 4°C until use. Sterile glucose (BDH Chemicals, UK) solution in mH$_2$O was prepared by passing it through a 0.2 μm filter, and 20 mM glucose was added before used.

**2.2.5.2 General solutions**

**β-mercaptoethanol:** for general use - electrophoresis purity (BioRad Laboratories, USA); for tissue culture use – tissue culture grade (Gibco®, USA)

**λ-DNA marker:** PstI/λ-DNA marker was prepared by incubating 20 μg λ-DNA (New England Biolabs, USA), 18 μL NEBuffer 3.1 (New England Biolabs, USA), 10U PstI (New England Biolabs, USA) and mH$_2$O up to 180 μL, at 37°C overnight. The digestion was ceased by heating at 80°C for 20 minutes and 20 μL of 6x DNA loading buffer was then added. The marker was stored at -20°C and 10 μL aliquots used on a DNA agarose gel.

**Acryl/Bis™ (29:1) solution:** Astral, Australia

**Agarose gel:** 1% (w/v) DNA grade agarose (Progen Industries, Australia) in 1x TAE buffer, dissolved by heating in a microwave.

**Ammonium persulphate (APS):** 10% (w/v) ammonium persulphate (Sigma-Aldrich, USA) in deionised water stored in dark at 4°C.

**Ampicillin:** 100 mg/mL ampicillin (CSL, Australia) in mH$_2$O water, filter sterilised.

**BCIP/NBT solution for detection of alkaline phosphatase antibodies:** BCIP®/NBT solution,
premixed (Sigma-Aldrich, USA).

**Bovine serum albumin (BSA), molecular biology grade:** 1 mg/mL (New England Biolabs, USA), stored at -20°C.

**Bradford reagent:** 100 mg Coomassie Brilliant G-250 (Sigma-Aldrich, USA) dye was dissolved in 50 mL 95% (v/v) ethanol, then mixed with 100 mL 85% (v/v) phosphoric acid, and brought up to 1 L with dH2O. The solution was stored at 4°C, and filtered through a 0.20 μm filter before use.

**Bromophenol blue:** 1% bromophenol blue (BDH Chemicals, USA) (w/v).

**Calcium chloride (CaCl₂):** 1 M CaCl₂ (BDH Chemicals, USA) filter sterilised.

**Chloroform:** 100% (v/v) chloroform (BDH Chemicals, USA).

**Chloroform: Isoamyl (CI):** 96% (v/v) chloroform (BDH Chemicals, USA), 4% (v/v) isoamyl alcohol (BDH Chemicals, USA).

**Coating buffer (ELISA):** 0.016 M Na₂CO₃ (BDH Chemicals, USA), 0.034 M NaHCO₃ (BDH Chemicals, USA), pH 9.6.

**Coomassie blue staining:**

- **Coomassie blue destain solution:** 10% (v/v) ethanol (BDH Chemicals, USA) and 10% (v/v) glacial acetic acid (Fisher Scientific, USA).

- **Coomassie blue stain solution:** 0.1% (w/v) Coomassie brilliant blue R-250 (Sigma-Aldrich, USA), 50% (v/v) methanol (BDH Chemicals, USA), 10% (v/v) glacial acetic acid (Fisher Scientific, USA).

**DNA loading buffer 6X:** 0.25% (w/v) bromophenol blue (BDH Chemicals, USA), 0.25% (w/v) xylene cyanol FF (Sigma-Aldrich, USA), 40% (w/v) sucrose (Sigma-Aldrich, USA), 10 mM Tris-HCl (pH 7.6).

**Deoxynucleotide (dNTP) solution mix:** 10 mM each of dATP, dGTP, dTTP and dCTP (New England Biolabs, USA).

**Dextrose:** 10% (w/v) in mH₂O (BDH Chemicals, USA). Filter sterilised.
**Ethanol**: 70% (v/v), 95% (v/v), 100% (v/v) analytic ethanol (BDH Chemicals, USA).

**Ethidium bromide (EtBr)**: A stock solution of 10 mg/mL EtBr (Sigma-Aldrich, USA).

**Ethylene diaminetetra-acetate (EDTA) buffer**: 0.25 M EDTA (Merck, Germany), pH 8.0.

**Formaldehyde**: Formaldehyde (37% w/v) (Sigma-Aldrich, USA).

**Glucose**: 20% in mH$_2$O (BDH Chemicals, USA), filter sterilised.

**Glycerol**: 100%, 50% (v/v) glycerol (BDH Chemicals, USA).

**Hydrochloric acid**: 32% (w/v) (Ajax Chemicals Ltd., Australia).

**Hydrogen peroxide (H$_2$O$_2$)**: 30% (w/v) H$_2$O$_2$ (BDH Chemicals, USA).

**Immobilised ion metal affinity chromatography (IMAC) solutions**:
- **Native Binding buffer**: 50 mM NaH$_2$PO$_4$, 0.5 M NaCl, 10 mM imidazole.
- **Washing buffer**: 50 mM NaH$_2$PO$_4$, 0.5 M NaCl, 20 mM imidazole.
- **Elution buffer**: 50 mM NaH$_2$PO$_4$, 0.5 M NaCl, 250 mM imidazole.

**Imidazole**: A 5 M Imidazole stock solution (Sigma-Aldrich, USA) was prepared and filtered through a 0.45 μm filter.

**Immunoblotting solutions**:
- **Blocking buffer**: 5% (w/v) skim milk in PBS
- **Antibody diluents**: 1% (w/v) skim milk in PBS
- **Washing buffer**: 1% (w/v) skim milk in PBST
- **TMB single solution**: Life Technologies, USA

**Isopropanol**: Propan-2-ol (BDH Chemicals, USA).

**Isopropyl-β-D-thiogalactopyranoside (IPTG) 1 M stock**: 0.2 g/mL IPTG (Thermo Scientific, USA) sterilised and stored at -20°C.

**Magnesium chloride**: BDH Chemicals, USA

**Methanol**: 100% (v/v) (BDH Chemicals, USA).

**Nickel Sulphate (0.2 M)**: Nickel sulphate (2.6 g) (BDH Chemicals, USA) was dissolved in 50 mL of mH2O.
Phenol (saturated): Supplied by Astral Scientific, Australia.


Phosphate-buffered saline (PBS): 1 tablet Dulbecco’s A PBS (Oxoid, UK) in 100 mL H₂O (sodium chloride 0.8%, potassium chloride 0.02%, disodium hydrogen phosphate 0.115% potassium dihydrogen phosphate 0.02%). Autoclaved if required.

PBST: PBS and 0.05% (v/v) Tween 20 (polyoxyethylene sorbitan monolaurate) (Sigma-Aldrich, USA).

Protease Inhibitor Cocktail (100X): mix of AEBSF, Aprotinin, Bestatin, E64, Leupeptin, and Pepstatin A (Cell Signaling Technology, USA)

Saline: 0.85% sodium chloride (BDH Chemicals, USA).

Sodium dodecyl sulfate: 10% (w/v) in mH₂O. Filtered through 0.45 μm filter

SDS-PAGE solutions:
- **Loading buffer (5x):** 60 mM Tris (pH 6.8), 25% (v/v) glycerol (BDH Chemicals, USA), 2% (w/v) SDS (BDH Chemicals, USA), 14.4 mM β-mercaptoethanol (BioRad Laboratories, Australia), 0.1% (w/v) bromophenol blue (Sigma- Aldrich, USA).
- **Separating gel (12.5%):** 375 mM Tris. HCl (pH 8.8), 0.1% w/v SDS, 12.5% v/v bis acrylamide with setting agents added prior to pouring: 0.06% w/v ammonium persulfate, 0.03% v/v TEMED.
- **Stacking gel (4.5%):** 125 mM Tris. HCl (pH 6.8), 0.1% (w/v) SDS and 4.5% bis acrylamide with setting agents added prior to overlaying of separating gel: 0.06% (w/v) ammonium persulfate and 0.03% (v/v) TEMED.
- **Running buffer stock (10x):** 0.25 M Tris, 1.92 M glycine (BDH Chemicals, USA), 1% (w/v) SDS (BDH Chemicals, USA). The pH was adjusted to 8.3.

Silver staining solutions:
- **Fixative solution:** 40% (v/v) methanol (BDH Chemicals, USA), 10% (v/v) acetic acid (BDH...
Chemicals, USA), 50% (v/v) mH₂O.

- **Washing solution**: 30% (v/v) absolute ethanol (BDH Chemicals, USA), 70% (v/v) mH₂O.
- **Reductant**: 0.02% (w/v) sodium thiosulfate (BDH Chemicals, USA) in mH₂O.
- **Silver reagent**: 0.2% (w/v) silver nitrate (Sigma-Aldrich, USA), 0.02% (v/v) formaldehyde (Sigma-Aldrich, USA) in mH₂O. Stored in dark at 4°C.
- **Developer**: 3.2 % developer reagent (BioRad Laboratories, USA) in mH₂O. Made for immediate use.
- **Stopping solution**: 5% (v/v) acetic acid (Fisher Scientific, Australia) in mH₂O.

**Skim milk**: Bonlac Foods Limited, Australia.

**Sodium acetate**: 3 M (BDH Chemicals, USA), pH 4.6.

**Sodium chloride**: 5 M stock solution of sodium chloride.

**Sucrose gradient for virus purification**:
- **TNE buffer**: 50 mM Tris (BDH Chemicals, USA), 140 mM NaCl, 5mM EDTA (BDH Chemicals, USA), pH7.4. Sterilisation by autoclave.
- **Sucrose gradient**: 15% and 60% high grade sucrose (BDH Chemicals, USA) in TNE buffer. Filtered through 0.2 μm filter and stored at 4°C.

**TCA**: 100% (w/v) Trichloroacetic acid (Calbiochem, Germany)

**TE buffer x10**: 100 mM Tris (BM Chemical, China), 10 mM EDTA (Amresco, USA), pH 8.0.

**TEMED (N,N,N.,N.-tetramethylethylenediamine)**: Electrophoresis purity (BioRad Laboratories, Australia).

**3, 3', 5, 5'-tetramethylbenzidine (TMB)**: for ELISA, TMB substrate reagent A (BD Biosciences, USA) and TMB substrate reagent B (BD Biosciences, USA) were mixed in a ratio of 1:1 and used within 10 minutes.

**Tris (hydroxymethyl) aminomethane (Tris)**: 2 M stock solution (Merck, Germany).

**Tris-Acetate buffer (TAE) x 50**: 2 M Tris (BDH Chemicals, USA), 1 M acetic acid, 0.1 M EDTA (BDH Chemicals, USA).
TRizol® LB reagent: Invitrogen, USA

Trypan Blue stain (0.4% (w/v)): Sigma-Aldrich, USA

2.2.5.3 Enzymes

All enzymes were used according to manufacturer’s instruction with their supplied buffer, and were stored at -20°C.

DNA polymerase, Taq DNA Polymerase with Standard Taq Buffer: 3U/μL (New England Biolabs, USA).

DNA Ligase (T4): 10U/μL (New England Biolabs, USA).

Trypsin-EDTA (1X): 0.05% with phenol red (Gibco®, USA).

2.2.5.4 Antibodies

Goat anti-chicken IgA-HRP: Abcam®, USA

Goat anti-mouse IgG H&L AP: Abcam®, USA

Goat anti-rabbit IgG H&L AP: Abcam®, USA

Goat anti-rabbit IgG (whole molecule)-HRP: Sigma-Aldrich, USA.

Mouse anti-6X His tag®: Abcam®, USA

Mouse anti-His (C-Term)-HRP: Life Technologies, USA

Rabbit influenza (A/Puerto Rico/8/34) antiserum: (provided by the WHO Collaborating Centre for Reference and Research on Influenza)

Rabbit anti-chicken IgG-HRP: Merck Millipore, Germany

Rabbit anti-mouse IgG (whole molecule)-HRP: Sigma-Aldrich, USA
2.3 Microbiological methods

2.3.1 Bacterial strains and plasmids

The description of all the bacterial strains used throughout this study is listed in Table 2.1, and description of all plasmid vectors developed throughout the study is listed in Table 2.2.

2.3.2 Bacterial storage

_E. coli_ and _Salmonella_ strains were stored at -80°C in 30% glycerol.

2.3.3 Culture conditions

All _E. coli_ and _Salmonella_ strains were cultured on solid microbiological media under aerobic conditions at 37°C for 16-19 hours. When broth cultures were used, the strains were grown aerobically at 37°C for 16-19 hours in an orbital shaking incubator (Edwards Instrument, Australia) set at 150 rotations per minute (rpm).
Table 2.1: Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Genotype/Description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> DH5-α</td>
<td>F’/endA1, hsdR17(r’k’m’t’), subE44, thi-1, recA1, gyrA (NalR), relA1, Δ(lacZYA-argF)U169 (m80lacZΔM15)</td>
<td>[321]</td>
</tr>
<tr>
<td><em>Salmonella Typhimurium</em> LT2-9121</td>
<td>leu hsdL trpD2 rpsL120 ilv52 metE551 metA22 hsdA hsdB</td>
<td>Prof. P. Reeves, Department of Microbiology, The University of Sydney</td>
</tr>
<tr>
<td><em>Salmonella Typhimurium</em> STM1</td>
<td>ΔaroA Δsec'</td>
<td>RMIT University [320]</td>
</tr>
</tbody>
</table>

Table 2.2: Plasmid vectors used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genotype/Description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR2.1</td>
<td>3.9 kbp PCR cloning plasmid, AmpR, KmR, lacZ</td>
<td>Invitrogen, USA</td>
</tr>
<tr>
<td>pMOhly1</td>
<td>11.9 kbp protein expression plasmid containing the <em>E. coli</em> α-haemolysin secretion system. Gene of interest was ligated into a truncated hlyA', immediately upstream of the C-terminus secretion signal and downstream of the initiation codon.</td>
<td>Dr. I. Gentschev [322]</td>
</tr>
<tr>
<td>pHES</td>
<td>4.9 kbp protein expression plasmid containing <em>Salmonella Typhimurium</em> LT-2 ShdA autotransporter gene. Gene of interest was ligated into the N-terminus of the ORF immediately after the signal peptide.</td>
<td>Dr. A. Fernandez [5]</td>
</tr>
</tbody>
</table>
2.4 Methods of RNA extraction and cDNA synthesis

2.4.1 RNA extraction

The protocol was adapted from the manufacturer’s instructions (Invitrogen, USA), with some modifications. All reagents and equipment were chilled to \(4^\circ\)C prior to commencement, and all centrifugation steps were performed at \(4^\circ\)C, unless otherwise stated. Equal volumes of purified PR-8 virus were mixed with TRIzol\textsuperscript{®} reagent and incubated at room temperature for five minutes, followed by the addition of one tenth volume of chloroform.

The reaction was vigorously mixed for 15 seconds and incubated for five minutes at room temperature. Following incubation, the samples were centrifuged at 12,000 \(\times\) \(g\) for 15 minutes, and the aqueous phase was collected for RNA precipitation. The RNA molecules were precipitated by the addition of an equal volume of isopropyl alcohol, and the samples were vigorously mixed and incubated at room temperature for five minutes. After incubation, the RNA molecules were pelleted by centrifugation at 12,000 \(\times\) \(g\) for 10 minutes. The RNA pellet was washed twice with 75% ethanol and air dried before being resuspended in DNase/RNase-free water. The isolated RNA samples were subjected to immediate cDNA synthesis.

2.4.2 cDNA synthesis

The procedure was performed according to the manufacturer’s instructions (Bioline, Australia), with some modifications. All reagents and buffers were provided in the kit except the DNase/RNase-free water. Briefly, a RNA priming reaction was prepared (as shown in Table 2.3). The reaction was incubated at 65\(^\circ\)C for 10 minutes, followed by incubation on ice for two minutes. During the incubation steps, a Reverse transcription mix was prepared (as shown in Table 2.4). Following the RNA-priming incubation, 10 \(\mu\)L of the primed RNA solution
was combined with 10 μL of reverse transcription mix and incubated at 42°C for one hour. The reaction was terminated by incubation at 70°C for 15 minutes and then chilled on ice. The newly synthesised cDNA molecules were subjected to immediate PCR amplification.
### Table 2.3: RNA priming solution

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA molecule</td>
<td>6.0 μg</td>
</tr>
<tr>
<td>Oligo (dT)$_{18}$</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>Random Hexamer</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>100 mM dNTP</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>DNase/RNase-free water</td>
<td>To a total volume of 10 μL</td>
</tr>
</tbody>
</table>

### Table 2.4: Reverse transcription mix

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X RT Buffer</td>
<td>4.0 μL</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>1.0 μL</td>
</tr>
<tr>
<td>Reverse Transcriptase (200U/μL)</td>
<td>0.25 μL</td>
</tr>
<tr>
<td>DNase/RNase-free water</td>
<td>4.75 μL</td>
</tr>
</tbody>
</table>
2.5 Methods of DNA analysis

2.5.1 Plasmid extraction

Plasmids used in restriction digests and sequencing were purified using the QIAprep® spin miniprep kit as per the manufacturer’s instructions with modification, in which, the elution buffer was replaced with DNase/RNase-free water and eluted using a total of 30 μL in two steps (20 μL of DNase/RNase-free water was first added directly onto the spin column membrane, and incubated for five minutes at room temperature before centrifugation, thereafter, a 10 μL DNase/RNase-free water was added as described, and incubated for 10 minutes prior centrifugation).

2.5.2 Extraction of DNA from agarose gel

DNA fragments were excised from the agarose gel and extracted using QIAquick gel extraction kit as per the manufacturer’s instructions with modification (as described in Section 2.5.1). This kit was also used for the purification of DNA ligation product as per the manufacturer’s instructions with modification (as described in Section 2.5.1).

2.5.3 Agarose gel electrophoresis

5 μL of PCR or extracted DNA fragment was mixed with 1 μL of 6X DNA loading buffer and separated on a 1-2% DNA grade agarose gel in a mini-gel or midi-gel unit filled with fresh 1X TAE buffer. A current of 90 V was applied to the gel, and DNA fragments separated for 1.5 hours. The gel was then stained in a 3 μL/mg EtBr bath for 20 minutes, followed by a 40 minutes destaining procedure in running tap water.

The DNA products were visualised with an UV illuminator and photographed with the Gel Doc imaging system (BioRad Laboratories, USA). Sizes of DNA fragments were estimated by
the additions of the PstI/λ-DNA marker to the gel before electrophoresis. A diagram of the λ-DNA sizes is provided in Appendix 1.

2.5.4 Spectrophotometric quantification of DNA

The LUMIstar Omega Microplate luminometer was used to estimate the optical density (OD) of a solution of DNA at a wavelength of 260 nm, the optimal wavelength for nucleic acids. Protein contamination was detected by an OD reading at 280 nm. The OD ratio of DNA/protein was determined to estimate the quality of the DNA. Ratio of 1.8-2.0 indicated good quality DNA [323]. An OD of 1 corresponded to 50 μg/mL double stranded DNA and the amount of DNA present was determined using this standard.

2.5.5 DNA quantification using DNA gel electrophoresis

PstI/λ-DNA marker was separated on an agarose gel along with sample DNA fragments. The quantity of DNA within each digested fragment of λ-DNA is provided in Appendix 1 and was compared with intensities of unknown DNA samples.

2.6 DNA manipulation

2.6.1 Restriction enzymes

The restriction enzymes (RE) used in the study are listed in Table 2.5. Approximately 1 μg of plasmid DNA was digested with 1 U of enzyme supplied with 1X buffer in a total volume of 20 μL in a 37°C water bath for three hours or overnight. When high purity DNA fragments were required for subsequent procedures (e.g. enzymatic ligation), enzymes were heat-inactivated to the manufacturer’s recommended temperature and duration. However, if the enzyme cannot be heat inactivated, QIAquick gel extraction kit was used to eliminate the enzymes as per manufacturer’s instruction. All enzymes were purchased from New England Biolabs, USA.
Table 2.5: List of restriction enzymes (RE) used in this study

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Recognition sequence</th>
</tr>
</thead>
</table>
| *Pvu*II            | 5’...CGATCG...3’  
|                    | 3’...GCATTG...5’    |
| *Eco*RI            | 5’...GAATTC...3’    |
|                    | 3’...CTTAAG...5’    |
| *Sbf*I             | 5’...CTCGAG...3’    |
|                    | 3’...GGACGTC...5’   |
| *Nsi*I             | 5’...ATGCAT...3’    |
|                    | 3’...TACGTA...5’    |
| *Pst*I             | 5’...CTCGAG...3’    |
|                    | 3’...GAACGTC...5’   |
2.6.2 DNA amplification

2.6.2.1 Primers

A list of primers used throughout the study is provided in Table 2.6. Primers were designed with the assistance of the software program Sci Ed Central compatible for Windows 7. In ideal situation, primers were designed to have guanine & cytosine (GC) content between 40-60% with a melting temperature (Tm°C) between 55-75°C. However, in the case of pMOhly1_SbfI_Rev, the GC content was compromised in the effort to incorporate a 6X His-tag for subsequent analysis. Primers were obtained as lyophilised samples from GeneWorks Pty. Ltd., Australia

2.6.2.2 Polymerase chain reaction (PCR) using DNA templates

General PCR was performed according to the polymerase manufacturer’s instructions (New England Biolabs, USA). The general reaction mix and programs was listed in Table 2.7 and Table 2.8. the annealing temperature is specified in the relevant chapters. Taq polymerase was used for all DNA amplification purposes.

The reagents were prepared aseptically. Either G-strom or GeneAmp PCR machine was used to amplify the DNA. Approximately 10 ng of DNA template was used for the reactions, and reactions were prepared in a final volume of 50 μL.

2.6.2.3 Colony PCR

A protocol applied to fast screening for plasmid inserts directly from E. coli colonies.

A small amount of colony was transferred into an eppendorf tube containing 50 μL of sterile PBS from freshly grown culture. The colony was resuspended using vortex mixer and incubated at 100°C for three minutes on a dry heating block. Following incubation, the
sample was chilled on ice and 2 μL of the treated colony suspension was used as DNA template. The reaction composition and cycling conditions remained the same as described in Section 2.6.2.2.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Description</th>
<th>Tm°C*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMOhly1_SbfI_For</td>
<td>5’-CGGCCTGCAGGCTGAAAGGCAAACCTACTGGTC-3’</td>
<td>Forward primer for amplification of influenza HA1 for pMOhly plasmid, with sbfI RE site (underlined).</td>
<td>71</td>
</tr>
<tr>
<td>pMOhly1_SbfI_Rev</td>
<td>5’-CGGCCTGCAGGATGGTATGGATGGATGCTTGGATTGAATGGACGGATTG-3’</td>
<td>Reverse primer for amplification of influenza HA1 for pMOhly plasmid, with sbfI RE site (underlined).</td>
<td>74</td>
</tr>
<tr>
<td>pHES_EcoRI_For</td>
<td>5’-CGCAATTCTAGGAAGGCACCTACTGGT-3’</td>
<td>Forward primer for amplification of influenza HA1 for pHES plasmid, with EcoRI RE site (underlined).</td>
<td>61</td>
</tr>
<tr>
<td>pHES_PvuI_Rev</td>
<td>5’-CGGCCATCGTGGATTGAATGGACGGATTGT-3’</td>
<td>Reverse primer for amplification of influenza HA1 for pHES plasmid, with PvuI RE site (underlined).</td>
<td>64</td>
</tr>
<tr>
<td>SEQF_pMOhly1</td>
<td>5’-GCTGGACTTCAGTGATCGTAATG-3’</td>
<td>Forward sequencing primer of pMOhly1 plasmid</td>
<td>59</td>
</tr>
<tr>
<td>SEQR_pMOhly1</td>
<td>5’-GCTGATGTGGTCAGGGTTATGGAG-3’</td>
<td>Reverse sequencing primer of pMOhly1 plasmid</td>
<td>57</td>
</tr>
<tr>
<td>SEQF_pHES</td>
<td>5’-TTTCCCGACTGGAAGCCCGGAGCTGAG-3’</td>
<td>Forward sequencing primer of pHES plasmid</td>
<td>64</td>
</tr>
<tr>
<td>SEQR_pHES</td>
<td>5’-CGGATTTCGCTTTACCGCTTTTG-3’</td>
<td>Reverse sequencing primer of pHES plasmid</td>
<td>59</td>
</tr>
<tr>
<td>SEQF_PR8HA1</td>
<td>5’-CCGGAAATAGCAGAAAGCCACCAAAG-3’</td>
<td>Forward sequencing primer of PR-8 HA1 gene</td>
<td>59</td>
</tr>
<tr>
<td>SEQR_PR8HA1</td>
<td>5’-AGCCCTCCTTCCGTCAGCCTAG-3’</td>
<td>Reverse sequencing primer of PR-8 HA1 gene</td>
<td>63</td>
</tr>
</tbody>
</table>

*Calculated by company of synthesis

Blue sequence indicating a sequence encoding for 6X-His tag
Table 2.7: Standard reaction conditions for amplification of DNA by PCR

<table>
<thead>
<tr>
<th>PCR reagent</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template</td>
<td>10-100 ng</td>
</tr>
<tr>
<td>dNTP</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Primer A</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Primer B</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>10X PCR buffer (with MgCl₂)</td>
<td>1X</td>
</tr>
<tr>
<td><em>Taq</em> polymerase</td>
<td>1 U/50 µL</td>
</tr>
</tbody>
</table>

Table 2.8: Standard amplification conditions of PCR with *Taq* polymerase

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (s)</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>120</td>
<td>1X</td>
</tr>
<tr>
<td>94</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Specific annealing  °C</td>
<td>60</td>
<td>35X</td>
</tr>
<tr>
<td>72</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>7 min</td>
<td>1X</td>
</tr>
</tbody>
</table>
2.6.3 Ligation

Ligations were performed with purified digested DNA at a ratio of either 1:3 or 1:6 (vector molecule to insert molecules) with the addition of 2 U T4 ligase and buffer in a total volume of 10 μL. The reaction was incubated at 14 °C overnight. The ligated DNA product was then purified using QIAquick gel extraction kit prior to transformation via electroporation, which is crucial to prevent arcing during electrotransformation.

2.6.4 Electrocompetent cell preparation

Bacterial cells were prepared for electrotransformation by the method supplied with the Gene Pulser apparatus user’s manual (BioRad Laboratories, USA) with minor adjustments. Briefly, 200 mL of Luria-Bertani (LB) broth was inoculated with 1 in 100 volume of an overnight bacterial culture. The cells were grown with vigorous shaking at 37 °C to an OD$_{600}$ of 0.5-0.7 (early to mid-log phase). The culture was cooled on ice for 30 minutes and the culture pelleted by 4,000 x g centrifugation for 10 minutes at 4 °C. The supernatant was discarded and the pellet resuspended in an equal volume of ice-cold sterile Milli Q water (mH$_2$O). The cell suspension was centrifuged at 4,000 x g for eight minutes at 4 °C. The cells were pelleted as above and resuspended in 4 mL ice-cold 10% glycerol. The cells centrifuged once again at 3,500 x g for six minutes at 4 °C, and resuspended with 10% glycerol in a final volume of 200 μL. 50 μL aliquots were loaded for immediate use of electrotransformation.

2.6.5 Electrotransformation

The method supplied with Gene Pulser apparatus user’s manual (BioRad Laboratories, USA) was applied for the high efficiency electrotansformation of _E. coli_ and _S. Typhymurium_. Freshly prepared electrocompetent cells were mixed with up to 2 μL of purified DNA and transferred to an ice-cold electroporation cuvette with a 0.2 cm gap. The pulse settings used
to deliver DNA into the cells were 2.4 kV, 25 μF and 200 Ω.

After the pulser, S.O.C medium (pre-warm to 37°C) was immediately added to the cells to a total volume of 1 mL and were incubated at 37°C with 50 rpm shaking for one hour. 50 μL, 100 μL and 200 μL of the transformed culture were plated out onto LB agar containing the appropriate selective agents. The remaining contents of transformed mixture were concentrated by centrifugation at 4,000 x g for five minutes and the cell pellet was plated onto LB agar containing the appropriate selective agents.

2.6.6 DNA sequence analysis

DNA sequence analysis was out-sourced from Australian Genome Research Facility, Melbourne. DNA samples were purified using QIAprep® spin miniprep kit and eluted in DNase/RNase-free water, the DNA quantity and quality were estimated using LUMIstar Omega Microplate luminometer. For double-stranded plasmid DNA, 600-1500 ng of purified DNA template was mixed with 9.6 pmol of primer in a total volume of 12 μL. The reaction mix was sealed in a 1.5 mL eppendorf tube, and sent to the facility via express post under ambient temperature. The sequencing result was analysed in Sci Ed Central software.

2.7 Protein methods

2.7.1 Preparation of whole cell lysates

Samples of E. coli and S. Typhimurium from broth cultures were pelleted by centrifugation at 16,000 x g for five minutes. The pellet was resuspended in one volume of 10mM Tris-HCl pH 8.0 (supplemented with Protease Inhibitor Cocktail (Cell Signaling Technology, USA)), and sonicated using Branson Digital Sonifier®, with six cycles of 15 seconds ON, 45 seconds OFF at 18% amplitude. Cell debris was pelleted by centrifugation and the protein content of the
supernatant was determined using the Bradford protein assay.

2.7.2 Bradford assay

Protein content determination was performed using the Bradford assay [324, 325]. Nine hundred microlitre of Bradford reagent was added to 100 μL of sample, or standards, mixed by vortexing and left to stand for two minutes. Samples and standards were diluted in filtered 0.15 M NaCl. Bovine serum albumin (BSA) was used as the standard protein solution. 200 μL of processed sample was added to the well of a 96-well microtitre plate. The OD of samples was measured with iMark™ microplate absorbance reader at 600 nm (OD$_{600}$), and a standard curve constructed by plotting the concentrations of the bovine serum albumin standards (μg) versus absorbance was used to determine the protein concentration of unknowns.

2.7.3 Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using a discontinuous buffer system [324]. The gels were prepared using SDS stacking gel buffer and SDS separating gel buffer. Proteins were mixed with 5X SDS loading buffer and heated to 100°C in a heating block for five minutes. The proteins were separated by electrophoresis at 80 V for 30 minutes and then 180 V for 50 minutes in an electrophoresis unit (BioRad Laboratories, USA) containing SDS running buffer. Three gels were run at the same time in this system. Two gels were transferred onto nitrocellulose membranes for immunoblotting, and the remaining gel was subjected to either silver or Coomassie blue staining. Protein markers were loaded together with other protein samples and used for the determination of relative protein mass.

For silver stained gels, either a Protein Marker, Broad Range (New England Biolabs, USA) (Appendix 2) or a Precision Plus Protein™ All Blue Standards (BioRad Laboratories, USA)
was used was loaded onto gels. For immunoblotting, either a ColorPlus™ Prestained Marker (New England Biolabs, USA) (Appendix 4) or a Precision Plus Protein™ Dual Color Standards (BioRad Laboratories, USA) (Appendix 5) or a Precision Plus Protein™ Kaleidoscope™ Standards (BioRad Laboratories, USA) (Appendix 6) was loaded onto gels (detail is specified in each relevant Figures).

2.7.3.1 Coomassie staining
SDS-PAGE gels were stained in Coomassie blue staining solution for 30 minutes. Gels were rinsed under dH₂O and destained in the Coomassie detaining solution for one hour. A tissue was folded and placed into the destaining solution to assist with the stain removal. Gels were then soaked in dH₂O and gently shaked overnight.

2.7.3.2 Silver staining
The SDS-PAGE gels were released into a flat-bottom glass tray and covered with Fixative solution for a minimum of 40 minutes or overnight. Direct contact with gel was definitely avoided. The gels were washed three times with the Washing solution, 20 minutes each. The gels were soaked in Reductant for one minute and rinsed with distilled water thoroughly (two minutes under running water), and soaked with Silver Reagent for 30 minutes. The gels were rinsed with distilled water again. The gel was then soaked in developer, and fresh developer was added when yellow precipitates appeared until protein bands reached the desired intensity. The gels were rinsed with running water for two minutes and soaked in Stop solution for at least 20 minutes.

2.7.4 Immunoblotting
Protein immunoblotting was performed after completion of SDS-PAGE (as described in Section 2.7.3) by the “iBlot™ Dry Blotting System” (Invitrogen, USA). The gel was placed
onto the (Anode) bottom stack with pre-placed nitrocellulose membrane, and a piece of filter paper was placed on top of the SDS-PAGE gel and the (Cathode) top stack was placed on top of the filter paper. A provided sponge was then loaded onto the lid of the iBlot\textsuperscript{TM} machine, and the lid was closed and initiated the protein transfer. Proteins were transferred onto the nitrocellulose membrane using the default program for seven minutes.

Following this the nitrocellulose membrane was removed and soaked in Blocking buffer for a minimum of one hour, then the membrane was soaked in primary antibody for minimum of two hours or overnight at 4\textdegree{}C. The primary antibodies were diluted to 1:6000 in Diluent.

The membrane was washed three times with Washing buffer, 20 minutes each, and then incubated with secondary antibody, which were diluted to 1:6000 in diluent for one hour in dark. The membrane was washed with Washing buffer three times, 20 minutes each. The membrane was developed with TMB (3, 3´, 5, 5´-tetramethylbenzidene) for HRP conjugated antibodies or BCIP/NBT solution for AP conjugated antibodies substrate until the desired signal intensity was reached. The membrane was gently rinsed with water to stop the development.

### 2.7.5 Enzyme-linked immunosorbent assay

Ninety-six well flat bottomed sterile plates (Greiner, Germany) were coated with 100 μL of 5 μg/mL purified protein sample diluted in PBS and incubated for one hour at 37\textdegree{}C or 4\textdegree{}C overnight. Unbound protein was removed with three washes in PBST (PBS and 0.05% (v/v) Tween 20) and the plate coated with 200 μL of blocking buffer. After a further hour incubation at 37\textdegree{}C, plates were washed as above, patted dry on absorbent paper towelling, and 100 μL of primary antibody two-fold serially diluted in diluent (PBST/1% (w/v) skim milk) was added. Plates were incubated at 37\textdegree{}C with 150 rpm shaking on an ELISA shaker platform.
for two hours. The solution was discarded, wells washed four times in PBST and patted dry. Secondary antibody conjugated to HRP was diluted 1:5000 in diluent and 100 μL added to the wells of the ELISA plate. Following a one hour incubation at 37°C on a shaking platform, the plates were washed four times in PBST followed by a wash in dH₂O and then patted dry on absorbent paper. TMB substrate was prepared and 100 μL added to all wells. The development of the reaction was allowed to proceed for up 30 minutes and the reaction was stopped with 50 μL of 1 M sulphuric acid. The OD₄₅₀ of the wells was determined in iMark™ microplate absorbance reader. The endpoint was determined as the dilution at which the OD₄₅₀ was three times the background level (OD≥0.2). All samples were tested in duplicate.

2.8 Mammalian cells and tissue culture

2.8.1 Cell type used in this study

Madin-Darby canine kidney cells (MDCK) derived from the kidney of an adult cocker spaniel (The cell was purchased from Sigma-Aldrich, Australia as part of the European Collection of Cell Cultures operated by Public Health England).

2.8.2 General maintainance

All tissue culture procedures were performed under the protection of class II Biosafety cabinet (Aura 2000 M.A.C. BIOAIR Instrument, Italy). The cabinet and relevant equipment were ethanol disinfected and exposed under UV light for 20 minutes before commencing any work. Details of tissue culture procedures are described in [326].

Cell monolayers were generally maintained in 25 cm² or 75 cm² tissue culture flasks (Greiner, UK) in complete medium (Dulbecco’s modified Eagle’s medium (DMEM) with GlutaMAX™ supplement (Gibco®, USA), 10% (v/v) heat-inactivated FBS (Gibco®, USA), 1X
Penicillin-Streptomycin (Gibco®, USA) and incubated in a humidified incubator at 37°C with 5% CO₂ supplement (New Brunswick, USA). Cells were trypsinised (Trypsin/EDTA Becton Dickinson Ltd., UK), and sub-cultured every four to seven days as necessary.

### 2.8.3 Trypsinising and subculturing cells from a monolayer

Medium was removed from the cell monolayer by decanting. Cell monolayer was gently washed twice with 37°C 1X DPBS (Gibco®, USA) to remove any residual FBS that may inhibit the action of trypsin. Appropriate volume of 37°C trypsin/EDTA solution (Gibco®, USA) was added to cover adhering cell layer, and incubated at 37°C for up to 10 minutes. Monolayer was checked every two to three minutes for detachment by tilt against light, and gently tapped to facilitate the process. Up to 5 mL of complete medium was added to stop trypsinising process, and Pasteur pipette was used to rinse the bottom of the flask with cell suspension to help dislodge any remaining adherent cells. The cell suspension was centrifuged at 700 x g for three minutes, and cell pellet was resuspended with an equal volume of complete medium. Appropriate volume of complete medium was added into a fresh flask, and inoculated with the cell suspension, and the flask was incubated in a humidified 37°C, 5% CO₂ incubator. Sub-confluent culture was fed after three to four days if necessary, by removing old medium and replacing with fresh 37°C complete medium. Passage of secondary culture when cell line became confluent by repeating steps outlined above, and continue to passage as necessary.

### 2.8.4 Cell stock freezing and resuscitation

Adherent cells were trypsinised, resuspended in the DMEM, and pelleted at 700 x g for three minutes. Cells were then resuspended in “freezing medium” (DMEM supplemented with 20% FBS and 10% DMSO (Santa Cruz Biotechnology, Inc., USA)), aliquoted into cryovials (Nalgene Company, USA), and frozen at -20°C for two hours, then -70°C for three days before
long-term storage in liquid N\textsubscript{2}. For resuscitation of cells, cryovials were rapidly thawed at 37\textdegree C before centrifugation at 700 x g. Cells were then resuspended in complete medium and inoculated into tissue culture flask with appropriate volume of medium, then incubated at 37\textdegree C with 5\% CO\textsubscript{2} overnight. Medium was replaced after 24 hours in order to remove traces of DMSO.

### 2.9 Virus propagation

#### 2.9.1 Virus strain used in this study

Influenza virus strain A/Puerto Rico/8/34 (H1N1) (PR-8) (stock provided by RMIT University).

#### 2.9.2 Virus cultivation in cell monolayer

The virus propagation protocol was adapted from the published protocol [327].

A confluent monolayer of MDCK cells were infected with virus diluted in DMEM at an appropriate multiplicity of infection. Prior to infection, the monolayer was washed twice with 37\textdegree C 1X DPBS to remove all traces of serum, and incubated with 1:1000 diluted virus stock (HA titre = 400). Following incubation at 37\textdegree C for one hour, the medium was discarded and washed once with DMEM (generally, 7 mL for 25 cm\textsuperscript{2} flask and 13 mL for 75 cm\textsuperscript{2} flasks). DMEM (supplemented with 0.2\% Bovine serum albumin fraction V solution (Gibco\textsuperscript{®}, USA)), was added and the cells were incubated at 37\textdegree C for up to four days, with regular observation for cytopathic effect (cell death). Incubation was stopped when 50\% of the monolayer cells were detached from the bottom. The flasks were frozen at -80\textdegree C until purification.

#### 2.9.3 Virus purification and storage

Virus infected cell monolayer was subjected to two cycles of freeze and thaw, and centrifuged at 5,000 x g for 10 mintues to removed cell debris. The supernatant was filtered
through 0.2 μm filter and the processed supernatant was gently loaded onto a two-layer sucrose gradient (15-60%). Sucrose gradient was prepared by loading 2 mL of 60% sucrose solution and overlaid with 2 mL of 15% sucrose solution in a Ultra-Clear™ thinwall tube (Beckman Coulter, Australia). The tubes were loaded into a Beckman Coulter SW 41 Ti rotor and centrifuged at 152,000 x g in a Beckman Coulter Optima L-80 XP Ultracentrifuge (Beckman Coulter, Australia) for 1.5 hours. After centrifugation, the gradient interphase was carefully removed and diluted to a final volume of 8 mL in TNE buffer, and loaded into a new Ultra-Clear™ thinwall tube for centrifugation as previously described. The virus pellet was resuspended in 50 μL TNE buffer and aliquoted into 10 μL fractions for storage at -80°C until required.

2.10 Mouse studies

Four to six week old female BALB/c mice were purchased from the Animal Resource Centre, Canningvale, Western Australia. Mice were acclimatised for a minimum of one week prior to the start of any experimentation.

Mice were bled from the retro-orbital vein using a capillary tube. Blood samples were separated by centrifugation at 5,450 x g for 10 minutes and the sera was collected and stored at -20°C until required for assays. More detailed methods will be described in the relevant chapter. More detailed methods are described in Chapter 3. This mouse study was approved by the RMIT Animal Ethics Committee under AEC# 0911.

2.11 Chicken studies

Newly broiler chickens were acquired from Inghams Farms, Pakenham, Victoria. The chicks were allowed to acclimatise for one week prior to the start of any procedure.
Chapter 2

Chicken were wing-tagged at seven days of age for identification. Chicken sera was collected at the end point of the trial after decapitation. Blood samples were separated by centrifugation at 5,450 x g for 10 minutes and the sera was collected and stored at -20°C until required for assays. More detailed methods are described in Chapter 5. The chicken study was approved by the RMIT Animal Ethics Committee under AEC# 1310.
Chapter 3

Vaccination Study Using

*P. pastoris* Expressed rHA1
3.1 Introduction

The influenza virus can cause seasonal epidemics and has been implicated in four well-documented human pandemics in the past [163, 198]. Furthermore, the emergence of the HPAIV H5N1 and recent outbreak of H7N9 in China highlights the potential threat of an imminent pandemic. The HPAIV (i.e. H5 and H7 subtype) has been known to cause recurrent outbreaks in the poultry industry, which have resulted in occasional spill-over infections into the human population. Molecular studies have revealed the co-existence of human and avian influenza preferential-tropism receptors in many animals including swine, chicken and humans [216].

The potential of virus hybridisation in such situation is high, and a virus reassortment may lead to the emergence of a pandemic novel influenza strain [328]. To date, the best prevention method for influenza infections is through immunisation with egg-based vaccines. However, such a vaccine production method is unable to rapidly respond to pandemic outbreaks from antigenically novel strains due to its time-consuming production, and possibility of an egg-shortage during an avian influenza strikes [253].

Alternative approaches for influenza vaccine production have been intensively investigated in recent years. Many approaches have been tested and evaluated, one such example is the use of in vitro expressed influenza antigen HA protein as an immunogen [170]. The employment of recombinant influenza HA as a vaccine antigen can potentially overcome the disadvantages of the currently used egg-based vaccine manufacture [272, 289], as the antigen can be recombinantly produced in cultures instead of eggs, and the HA gene can be isolated soon after the outbreak of a newly circulating strain [329]. The influenza (HA) surface-glycoprotein is known to play an important role in the initial viral attachment and host cell endocytosis, and a significant population of HA-specific antibodies are responsible
for the inhibition of HA binding and/or fusion [241, 243]. HA1 is known to possess the major antigenic targets for these neutralising antibodies that inhibit viral attachment to target cells, and most of these antigenic sites are more liable to antigenic variation than the rest of the protein due to immune selection pressure [228, 330]. Therefore, the HA1 protein makes a promising candidate in vaccine developments for the prevention of influenza infection.

*Pichia pastoris* (*P. pastoris*) is a well-established eukaryotic expression system that has a number of advantages over the prokaryotic expression system in regards to the production of recombinant vaccine antigens [331], including: 1) rapid production; 2) well characterised expression-vectors and easy genetic manipulation steps; 3) diverse post-translational modifications including glycosylation, peptide-folding, and targeting secretion; 4) simple purification steps from engineered secretion proteins [290, 291, 332]. Among these advantages, post-translational protein glycosylation is considered the most important, because it facilitates the correct peptide folding and virus-host receptor recognition [333-335].

In my previous study, a codon-optimised gene sequence encoding for HA1 derived from influenza (A/Puerto Rico/8/34(H1N1)) was synthesised by GenScript USA Inc. (Figure 3.1), and inserted into the yeast vector pPICZα-A (Figure 3.2 and Figure 3.3), a vector equipped with a methanol inducible promoter.
Figure 3.1: The HA1 gene sequence synthesised by GenScript USA Inc.,

The gene has two restriction site inserts *EcoRI* & *XbaI* (in Blue), and two additional cytosine bases (in Red) were added to ensure that the protein is “in-frame”. The total length of this HA1 sequence was 992bp.
Figure 3.2: The in silico construct developed in SECental™ Clone Manager.

```plaintext
mrfpsiftav lfaassalaa pvnttttedet aqipaeavig ysdlegdfdv
avipfsnstn ngllfintti asiaakeegv slkreaaeae fdticigyha
nnstdtvdvtv leknvtvths vnlledshng klcrklgiap lqlgkcniaag
wllgnpecdp lllpvrswsyi vetpnsengi cypgdfidye elreqlssvs
sferfeifpk esswpnhntn gvtaacsheg kssfyrnnlw ltekegsypk
lknsyvnnkg kevlvlwgh hppnskeqqn lyqnenayvs vvtsnsynrf
tpediaerpkv rdqagrmnny wtllkgdtdi ifeangnlia pmyafalsrg
fgsiiitsna smhecntkcg tplgainssl pyqnhpvti gecpkyvrsa
klrmtglrn npsiqsrple qkliseedln savdhhhhhh
```

Figure 3.3: The protein sequence expressed from the pPICZα-A construct containing HA1 gene.

The protein sequence in **Blue** was the α-factor signal that is incorporated in the pPICZα-A vector and is responsible for protein secretion. The sequence in **Purple** is the rHA1 protein sequence, and the 6X-His tag is coloured **Red**.
The pPICZα-A construct was then incorporated into the *P. pastoris* GS115 genome via homologous recombination between the transforming DNA and regions of homology (*AOX1* gene) within the genome [336, 337]. Successful transformation was confirmed using PCR (Figure 3.4), and recombinant influenza HA1 was detected as a secretory protein in a pilot expression study in a methanol induced culture (Figure 3.5).

In this study, the expression of rHA1 was optimised and rHA1 was expressed and purified by IMAC. An animal trial was then carried out to examine the immunogenicity of the rHA1 protein.

The work described in this chapter was as follows:

- To optimise the expression of rHA1 protein in *P. pastoris* by using different media and expression time.
- To purify rHA1 using IMAC and to analyse the protein using techniques including SDS-PAGE, immunoblotting with 6x-His antibody and PR-8 antiserum, and deglycosylation.
- To evaluate the immune responses to the purified rHA1 in a mouse trial.
Figure 3.4: The PCR amplification analysis on the DNA construct that has been transformed into the *P. pastoris* chromosomal DNA.

Lane M: *Pst*I/λ-DNA marker, Lane 1: chromosomal DNA of non-transformed *P. pastoris* GS115, Lane 2: PCR product of chromosomal DNA of non-transformed *P. pastoris* GS115, Lane 3: chromosomal DNA of putative clone, Lane 4, PCR product of chromosomal DNA of the putative clone. The information of primer sequences and PCR conditions were provided in EasySelect™ *Pichia* Expression Kit (Life Technologies, USA), designed to amplify the AOX1 gene. In the case of successful chromosomal insertion of the HA1 gene, a PCR product of approximately 1.5 kbp should be amplified, indicating the insertion of HA1 gene. As shown, the PCR confirmed the chromosomal insertion.
Figure 3.5: The silver stained SDS-PAGE gel of protein expression samples from BMMY medium.

Lane M: Protein Marker, Broad Range, Lanes 1 to 3: non-transformed *P. pastoris* GS115 at 0, 48 and 96 hours after expression, respectively, Lanes 4 to 6: transformed *P. pastoris* clone at 0, 48 and 96 hours after expression, respectively. The SDS-PAGE result showed that the rHA1 was expressed; as the expression medium of the clone appeared to have an additional protein-smear at approximately 80 kDa in molecular weight with an increasing intensity along the time-course of protein expression, which was not observed from the non-transformed *P. pastoris*. Furthermore, the rHA1 appeared as smears on the SDS-PAGE, indicating that the protein was hetero-glycosylated.
3.2 Materials and methods

3.2.1 General solution and media

3.2.1.1 Buffer solution for media

1 M potassium phosphate buffer, pH 6.0: prepared by combining 132 mL of 1 M K$_2$HPO$_4$ (BDH Chemicals, USA), 868 mL of 1 M KH$_2$PO$_4$ (BDH Chemicals, USA) and pH was adjusted using phosphoric (Sigma-Aldrich, USA) acid or potassium hydroxide (Sigma-Aldrich, USA).

3.2.1.2 Growth and expression media for P. pastoris

**Minimal dextrose medium + histidine (MDH):** 1.34% (w/v) Yeast Nitrogen Base (YNB) (Invitrogen, USA), 4 x 10$^{-5}$% (w/v) biotin (Sigma-Aldrich, USA), 2% (w/v) dextrose (BDH Chemicals, USA). All stock solutions were made separately and filter sterilised. mH$_2$O was autoclaved and cooled before addition of stocks. In the case of protein induction, 0.5% (v/v) methanol was added prior to inoculation of pre-expressed *Pichia* culture, and supplemented with 0.25% (v/v) methanol every 12 hours during expression.

**Minimal methanol medium + histidine (MMH):** 1.34% (w/v) YNB (Invitrogen, USA), 4 x 10$^{-5}$% (w/v) biotin (Sigma-Aldrich, USA), 0.5% (v/v) methanol (BDH Chemicals, USA). All stock solutions were made separately and filter sterilised. mH$_2$O was autoclaved and cooled before addition of stocks. 0.5% (v/v) methanol was added prior to inoculation of pre-expressed *Pichia* culture, and supplemented with 0.25% (v/v) methanol every 12 hours during expression.

**Buffered minimal glycerol medium (BMMH):** 1.34% (w/v) YNB, 4 x 10$^{-5}$% (w/v) biotin, 1% (v/v) glycerol (Sigma-Aldrich, USA), 0.5% (v/v) methanol (BDH Chemicals, USA), 100 mM potassium phosphate buffer pH 6.0. The medium was prepared by autoclaving mH$_2$O and
cooled before the addition of other supplements. 0.5% (v/v) methanol was added prior to inoculation of pre-expressed *Pichia* culture, and supplemented with 0.25% (v/v) methanol every 12 hours during expression.

**Buffered methanol-complex medium (BMMY):** 1% (w/v) yeast extract (Oxoid, UK), 2% (w/v) peptone (Oxoid, UK), 1.34% (w/v) YNB, 4 x 10^{-5}% (w/v) biotin, 1% (v/v) glycerol (Sigma-Aldrich, USA), 0.5% (v/v) methanol (BDH Chemicals, USA), 100 mM potassium phosphate buffer pH 6.0. The medium was prepared by autoclaving mH$_2$O containing 1% yeast extract and 2% peptone, and cooled before the addition of other supplements. 0.5% (v/v) methanol was added prior to inoculation of pre-expressed *Pichia* culture, and supplemented with 0.25% (v/v) methanol every 12 hours during expression.

### 3.2.2 rHA1 expression in *P. pastoris*

The clone was incubated for 40 hours in the MDH prior to expression, and protein-expression was induced by changing the carbon source from dextrose to methanol to up-regulate the *AOX1* methanol-utilisation gene, and thus express the recombinant protein.

#### 3.2.2.1 Optimisation of rHA1 expression

MMH, BMMH and BMMY expression medium were prepared in fresh and 100 mL of each of these expression medium was inoculated with 10 mL of the 40 hours-old cell culture. Four clones and one non-transformed *P. pastoris* GS115 were included in the pilot expression. All cultures were incubated at 30°C with 300 rpm shaking, 100% methanol (filter sterilised) was used to make up the final concentration of 0.5% (v/v) methanol every 12 hours, and 1 mL of expression medium were collected at 12 hours time intervals. All samples were snap-frozen with liquid N$_2$, and stored at -80°C for analysis.
3.2.2.2 Large-scale protein expression

Twenty milliliters of 40 hours-old *P. pastoris* clone culture was centrifuged at 2,500 x g for five minutes, and the cell pellet was inoculated into 200 mL of BMNNY expression medium in a 2 litre baffled flask. The culture was incubated at 30\(^{\circ}\)C and 300 rpm. Filter sterilised 100% methanol was added to the culture to the final concentration of 0.5% (v/v) every 12 hours for 84 hours. After expression, the culture was centrifuged at 4,500 x g for five minutes to remove yeast cells, and supernatant was snap-frozen with liquid nitrogen and stored at -80\(^{\circ}\)C until protein purification.

3.2.3 Purification of rHA1 using IMAC

Five milliliters of chelating Sepharose fast flow\(^{\circ}\) resin was loaded into a 10 mL propylene column (QIAGEN, Australia). The column was washed with 5 column volumes (CV) of sterile mH\(_2\)O, and loaded with 0.5 CV of 0.2 M NiSO\(_4\). The column was then washed with 5 CV of sterile mH\(_2\)O to remove unbound Nickel ions and then equilibrated with at least 5 CV of Native Binding Buffer.

Protein sample (expression medium) was filtered with 0.45 \(\mu\)m filter, and 8 mL of sample was loaded into the column and continuously rotated for 20 minutes to allow the His-tagged protein to bind to the Nickel ion. The column was placed in an upright position to allow resin to settle, and the sample was subsequently allowed to flow through. The same amount of sample was loaded and the process above was repeated for up to four times. The column was then washed with 15 CV of Native Wash Buffer, and loaded with 5 CV of Native Elution Buffer. The first 1 mL flow through was discarded since this is considered as the “dead volume” in the column, and the remaining flow through was collected for either immediate buffer exchange or stored at -80\(^{\circ}\)C until use.
3.2.3.1 Cleaning and regeneration of column

IMAC columns were re-used up to five times and cleaned between each use to remove hydrophobic and ionic bound contaminants. Column cleaning involves the application of 10 CV of 2 M NaCl, followed by 10 CV of 1 M NaOH, and finished with 10 CV of 70% ethanol (v/v). Columns were washed with dH2O between each solution and Ni²⁺ was stripped with 2 CV 0.05 M EDTA, 0.5 M NaCl, pH 7.0. A 20% ethanol solution was added to the sepharose when stored for later use.

3.2.4 Buffer exchange and protein concentration

Eluted proteins were concentrated using Centricon® centrifugal device (Milipore, Australia) with a cut-off pore size of 30 kDa according to the manufacturer’s instructions. Briefly, 4 mL of DNase/RNase-free water was added to the Centricon® centrifugal device and centrifuged at 4,500 × g for 15 minutes, and the flow-through discarded. 20 mL of protein sample was loaded into the device, and the tubes were centrifuged at 4,500 × g until the sample has been reduced to 1/100 of its original volume. Thereafter, sterilised PBS was added to top-up the sample volume to 20 mL and centrifuged again as described above, and the process was repeated three more times. The sample volume was reduced to a minimum and stored at -80°C until use. Salts and metals were removed by continual concentration and dilution of the protein sample in the new buffer.

3.2.5 Quantification of expressed rHA1

Quantification of purified protein was done using the Bradford assay (Section 2.7.2) and a spectrophotometric measurement at 600 nm on an iMark™ microplate absorbance reader. After quantification, the integrity of the protein was confirmed via SDS-PAGE gel.
3.2.6 Deglycosylation analysis

Glycosylation of rHA1 was evaluated by using Protein Deglycosylation Mix (New England Biolabs, USA), and examined using SDS-PAGE with silver staining and immunoblotting. The protocol used was derived from the Protein Deglycosylation Mix user guide (2009) (New England Biolabs, USA). The reaction composition were varied to examine the deglycosylation efficacy; 1 U enzyme equals to 5 μL of the deglycosylation enzyme mix in the total of 50 μL reaction; whereas 2 U equal to 10 μL of the enzyme mix in total of 50 μL reaction with reduced amount of H₂O added accordingly. Both native and denaturing conditions were available for the reaction. However, the deglycosylation enzymes were less effective to remove the additional oligosaccharide chains under native reaction conditions.

3.2.7 SDS-PAGE and Immunoblotting

SDS-PAGE gels subjected to silver staining and immunoblotting was used to examine the rHA1 protein integrity. SDS-PAGE gels were loaded with purified rHA1 and samples were separated together in the same electrophoresis system. After electrophoresis, gels were silver stained (as described in Section 2.7.3.2), or transferred onto nitrocellulose membrane for immunobloting using “mouse anti-His (C-term) antibody (Life Technologies, USA)” and “rabbit influenza (A/Puerto Rico/8/34 antiserum) (WHO)” (PR-8 antiserum). The immunoblotting procedure is as described in Section 2.7.4 with some modifications.

For immunoblotting with anti-His (C-term) antibody, the primary antibody was diluted 1:5000 in diluents and then incubated with the nitrocellulose membrane at room temperature for two hours in dark on a roller mixer (Ratek, Australia). The secondary antibody used was rabbit anti-mouse IgG-HRP (Sigma-Aldrich, USA) diluted 1:5000 in diluents, and the assay was developed using TMB single solution (Life Technologies, USA).
For the immunoblot assay with PR-8 antiserum, the primary antibody was diluted 1:3000 in diluents, and secondary antibody was goat anti-rabbit IgG-HRP (Sigma-Aldrich, USA) diluted 1:6000, and the assay was developed using TMB single solution (Life Technologies, USA).

### 3.2.8 Mouse vaccine trial using rHA1 vaccine candidate

The animal experimentation and relevant protocol were approved by the RMIT Animal Ethics Committee (AEC#0911). BALB/c mice was chosen to be the study model for this trial because there are predicted MHC class I and MHC class II epitopes for T-lymphocytes in BALB/c mice for HA1. The predictions were carried out using epitope prediction servers including:

- “SYFPEITHI: Epitope prediction” (http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm).

Two groups of five mice (five to seven week old female BALB/c mice) were immunised three times subcutaneously at days 7, 21 and 35 (around the flank region skin fold of dorsal area) using a 1 mL syringe and 23 or 25 gauge needle (depending on the viscosity of the mixture) with 100 μL of protein:adjuvant emulsion, which consists of 25 μg of protein. FCA (Frenud’s Complete Adjuvant) was used for the first injection, and FIC (Frenud’s Incomplete Adjuvant) was used for the subsequent two booster injections. The groups consisted of rHA1:Freund’s adjuvant and PBS:Freund’s adjuvant. The group administered with PBS:Freund’s adjuvant served as the control. Serum samples were collected three weeks after the final vaccination for the evaluation of humoral responses. The mice were killed by cervical dislocation and spleens were collected for immediate analysis of cell-mediated immune responses. In instances where organs were not required for further analysis, mice were killed by intraperitoneal (i.p.) administration of an overdose of Lethabarb (Euthanasia injection, 325
mg/mL Pentobarbitone Sodium) (Virbac-Animal Health, Australia).

3.2.8.1 Vaccine preparation

Prior to vaccinations, the rHA1 was prepared in an equal volume of FCA (Merck Millipore, Australia) for the initial injection, and FIA for the subsequent two booster injections. Briefly, an emulsion was prepared by drawing up and expelling equivalent volumes of protein and adjuvant through a 21 gauge needle in a 3 mL glass syringe until the fluidic suspension increased markedly in viscosity and was no longer able to disperse when a drop was placed in a glass beaker of cold water. Vaccine prepared in adjuvant was used within three hours of preparation.

3.2.9 Immunological assays for the mouse trial

3.2.9.1 ELISA protocols

Antibody responses of vaccinated mice were measured using the ELISA outlined in Section 2.7.5 with some modifications. Wells were coated with 10 μg/mL of purified rHA1 in ELISA coating buffer. Four-fold dilutions (starting at 1/50 dilution and serial diluted 11 times) of serum samples acquired from vaccinated mice were used as the primary antibody, and horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich, USA) (diluted 1:5000 in PBST/1% (w/v) skim milk) was used as the secondary conjugated antibody. The assay was developed using the substrate TMB (Life Technologies, USA), plates were incubated for 15 minutes before the addition of 50 μL of a 1 M sulphuric acid solution. The endpoint was determined as the dilution at which the optical density (OD) at a wavelength of 450 nm (OD$_{450}$) was three times the background level.
3.2.9.2  ELISpot assay

3.2.9.2.1  Media

**RPMI/NCS**: RPMI 1640 (Gibco®, USA) with 5% (v/v) heat-inactivated Newborn Calf Serum (NCS) (Gibco®, USA).

**RPMI/NCS/Abs**: RPMI/NCS with 0.1% (v/v) Penicillin-Streptomycin (Gibco®, USA).

3.2.9.2.2  Buffers and solutions

**ACK lysing buffer**: 0.15 M NH₄Cl (BDH Chemicals, USA), 10 mM KHCO₃ (Sigma-Aldrich, USA), 0.1 mM Na₂EDTA (Sigma-Aldrich, USA) dissolved in mH₂O and adjust pH to 7.3 using HCl (Ajax Chemicals Ltd., Australia). Filter sterilised.

**Antibody diluents**: PBS with 1% (v/v) NCS.

**ELISpot blocking buffer**: 5% (v/v) NCS in PBS.

**ELISpot coating buffer**: 35 mM NaHCO₃ (BDH Chemicals, USA), 11 mM Na₂CO₃ (BDH Chemicals, USA), pH 9.6.

**Concanavalin A**: 100 μg/mL in RPMI/NCS/Abs (Sigma-Aldrich, USA).

3.2.9.2.3  Antibodies

**Interferon-gamma (IFN-γ) (capture antibody)**: BD Pharmingen, USA.

**Interleukin-4 (IL-4) (capture antibody)**: BD Pharmingen, USA.

**Biotinylated detection antibody**: BD Pharmingen, USA.

**Alkaline phosphatase-labeled streptavidin**: Sigma-Aldrich, USA.

3.2.9.2.4  Equipment

**Cell strainer**: BD Falcon™ cell strainer (100 μm) (BD Biosciences, USA).

**ELISpot plate**: purchased from Millipore, USA.
3.2.9.2.5 ELISpot protocol

After cervical dislocation, mouse spleen cells were isolated for ELISpot assay. The protocol was adapted from a published method [4]. Briefly, spleens collected were crushed and resuspended in 5 mL of RPMI/NCS/Abs and filtered through a cell strainer. The cells were recovered by centrifugation at 750 x \( g \) for five minutes, and resuspended in 5 mL of ACK lysing buffer for depletion of red blood cells. After five minutes of incubation, 5 mL of RPMI/NCS/Abs was added and the cells recovered by centrifugation as described above. The splenocytes were washed three times with RPMI/NCS, and resuspended in RPMI/NCS. The number of viable cells was estimated using the Countess® Automated Cell Counter (Invitrogen, USA), by mixing a small volume of cells suspension with equal volume of Trypan Blue 0.4% staining solution (Sigma-Aldrich, USA), and loaded into a Countess® Cell Counting Chamber Slide (Invitrogen, USA) for viable cell estimation.

Before the day of the assay, the ELISpot plate was prepared by wetting the membrane with 100% methanol for five minutes, followed by three washes in sterile PBS. Capture antibodies were diluted in ELISpot coating buffer and added to the wells with the amount of 100 \( \mu \text{L/well} \) (IL-4 was diluted to 0.5 \( \mu \text{g/mL} \) and IFN-\( \gamma \) was diluted to 1.0 \( \mu \text{g/mL} \)). The plates were incubated overnight at 4\( ^\circ \)C. On the following day, the capture antibodies were discarded, and wells were washed three times with PBST. The wells were incubated with ELISpot blocking buffer for three hours. Prior to the addition of splenocytes, the wells were washed three times with 200 \( \mu \text{L} \) of PBST.

Prepared splenocytes were seeded in five wells at 1 x \( 10^6 \) cells in 90 \( \mu \text{L} \) of media per well. 10 \( \mu \text{L} \) of rHA1 protein was added at a concentration of 250 \( \mu \text{g/mL} \) in triplicate. 10 \( \mu \text{L} \) of RPMI/NCS/Abs was added in one well as an internal negative control, and 10 \( \mu \text{L} \) of Concanavalin A (100 \( \mu \text{g/mL} \)) solution was used as a positive control.
The plates were incubated for 20 hours at 37°C supplemented with 5% CO₂. After incubation, the splenocytes were discarded and the wells were washed seven times with 300 μL of PBST and twice with sterile mH₂O. The biotinylated detection antibody was diluted in antibody diluents to a final concentration of 0.5 μL/mL (v/v) and wells were incubated with 100 μL of the diluted antibody for two hours at room temperature. The plates were then washed six times using PBST and 100 μL of 1:1000 diluted alkaline phosphatase-labeled streptavidin was added to the wells. The plates were incubated for two hours at room temperature in the dark. After incubation, the antibody solution was discarded, and wells washed seven times with PBST and three times with PBS. The assay was developed by adding alkaline phosphatase substrate BCIP/NBT solution (Sigma-Aldrich, USA) (50 μL in each well), and incubation at room temperature in the dark until blue spots developed (30 minutes). The reaction was then stopped by three washes of dH₂O, and the plates air-dried before analysis. The spots were enumerated by visualisation under a dissecting microscope.

3.2.9.3 Hemagglutination Inhibition assay

3.2.9.3.1 Hemagglutination assay

A hemagglutination (HA) assay was carried out to estimate the virus titre of the stock, which is a prerequisite step for performing hemagglutination inhibition (HI) assay [338, 339].

One hundred microlitres of PBS diluted virus sample was added into the first well of a V-bottom-shaped 96-well plate, and a two-fold serial dilution was performed throughout the same row, resulting in 50 μL of serial diluted virus samples across the row.

Human erythrocytes (purchased from CSL Limited, Australia) were washed twice with sterile PBS and diluted to 0.75% (v/v). An equal volume of 0.75% human erythrocytes was then
added to all wells and incubated for one hour at room temperature. The ability of influenza HA to bind to the sialic acid receptors on the erythrocytes prevents these cells from settling to the V-shaped bottom. The HA unit was expressed as the highest virus dilution at which no hemagglutination was observed (Figure 3.6).

3.2.9.3.2 Hemagglutination Inhibition assay

The HI assay was used to evaluate viral-neutralising antibody responses elicited in immunised mice using PR-8 virus and human erythrocytes (CSL Limited, Australia) according to the WHO, 2011. Briefly, 50 μL of PBS diluted mouse serum was added into the first well of a v-bottom-shaped 96-well plate, and a two-fold serial dilution in PBS was performed, resulted in 25 μL of diluted serum across all wells. 4 HA units of PR-8 virus was added to each well and incubated at room temperature for 30 minutes. An equal volume of 0.75% human erythrocytes in PBS was then added to all wells and incubated for another hour. The HI titers were expressed as the logarithm of the reciprocal of the highest serum dilution at which no hemagglutination was observed, expressed as log₂ value (Figure 3.7).

3.2.10 Statistical analysis

All statistical analysis was calculated using the Microsoft Excel 2007 with the Data Analysis add-in. The results displaying a normal distribution and equal variances were analysed using Student’s T-test.
Figure 3.6: An example of hemagglutination assay.

Well 1: 1:100 PBS diluted PR-8 virus, wells 2-12: two-fold serially diluted virus sample. The HA unit was the dilution factor of the last well exhibiting complete hemagglutination, which was the forth well (1:400). Therefore, 1 HA unit was expressed as 400.

Figure 3.7: An example of hemagglutination inhibition assay.

Well 1: 1:50 PBS diluted mouse serum, well 2-12: serial diluted mouse serum sample.

The HI titre was the highest serum dilution at which no hemagglutination was observed, expressed as log₂ value. As such, the last well showing no hemagglutination was the third well (1:200 dilution), which is equivalent to 7.64 log₂.
3.3 Results

3.3.1 Optimisation of rHA1 expression

In comparison of the silver staining and immunoblotting on the expressed protein samples of different expression medium, BMMY was the medium which gave the best protein expression level; whereas the MMH expression medium was not optimal for the recombinant-protein expression (as shown in Figure 3.8). An estimation of rHA1 concentrations present in each growth medium was carried out using samples collected after 96 hours post-induction. Bradford assay revealed an estimated 1.57 mg/L, 2.02 mg/L and 2.15 mg/L in MMH, BMMH and BMMY media, respectively.

3.3.2 Time-course study on rHA1 expression

The BMMY appeared to be the optimal medium for the expression of rHA1, and thus a time-course study of the protein expression level in BMMY was carried out. As shown with the SDS-PAGE gel and immunoblot depicted in Figure 3.9, the concentration of the rHA1 gradually increased throughout the course of the protein expression, and the highest rHA1 concentration was reached between 72 hours and 96 hours after expression. Therefore, 84 hours of protein expression was used for the scale-up protein expression and further studies.
Figure 3.8: Silver-stained SDS-PAGE and immunoblotting results from *P. pastoris* expressing rHA1 using different media.

Three different yeast expression media were used to optimise protein expression, which includes MMH, BMMH and BMMY, each sample was harvested from expression medium after 96 hours of the initial induction. Lane M: ColorPlus™ Prestained Protein Marker, Lanes 1, 3, 6 and 7 were silver stained SDS-PAGE gels, and 2, 4, 6 and 8 were immunoblots against PR-8 antiserum. Lane 1 and 2 were the non-transformed yeast culture grown in BMMY for 96 hours as negative control. The results indicated that the maximum rHA1 yield was achieved in BMMY expression medium.
Figure 3.9: Time-course protein expression using BMMY medium.

A) Silver stained SDS-PAGE gel, and B) Immunoblot assay with PR-8 antiserum. Lane M: Protein Marker, Broad Range for A) and ColorPlus™ Prestained Protein Marker for B), Lanes 1 to 9: expression medium collected at 12 hour intervals (0, 12, 24, 36, 48, 60, 72, 84 and 96 hours after expression). Highest rHA1 expression reached after 72 hours of expression.
3.3.3 Protein purification and analysis

Large-scale protein expression was performed to generate large quantities of rHA1 for protein analysis and vaccine purposes. Immobilised Metal Affinity Chromatography (IMAC) with Nickel ions was used for protein purification, and successful purification of rHA1 was achieved (as shown in Figure 3.10) using the method described in Section 3.2.3. The purified rHA1 was deglycosylated with Protein Deglycosylation Mix (New England Biolabs, USA) to cleave off the oligosaccharide chains, and thus resulted as a clear protein band on the SDS-PAGE gel and immunoblot for estimation of the actual molecular weight of the rHA1 (as shown in Figure 3.11). Bradford assay estimated that the total rHA1 yield in the optimised protein expression was approximately 2.9 mg/L, and the Centricon ® Filter Device with 30 kDa cut-off was used to remove Imidazole and also concentrate the protein samples. Following buffer exchange, another Bradford assay was carried out to estimate the rHA1 content in solution, and the result estimated that the protein concentration of buffer exchanged products was approximately $6.2 \times 10^3$ mg/L.

Deglycosylation of the purified rHA1 was carried out to examine the true molecular weight of the protein. Both native and denaturing deglycosylation-reactions conditions were examined, and different amount of deglycosylation enzymes were included to examine the efficacy of deglycosylation reactions. As shown in Figure 3.11, the protein smear became a sharp band with corresponding molecular weight approximately 45 kDa. The molecular weight was approximately 3 kDa lower than what was predicted from ExPASy Proteomics Server. However, this small variation could be caused by inaccurate protein migration along the SDS-PAGE.
Figure 3.10: SDS-PAGE and immunoblotting results of IMAC purified rHA1 protein.

A) Silver stained SDS-PAGE gel, B) The immunoblot using Mouse anti-His (C-Term) antibody, C) The immunoblot using PR-8 antiserum; Lane M: ColorPlus™ Prestained Protein Marker, Lanes 1 to 3: 1st to 3rd elutions from IMAC column (the elutions were 1 mL fraction). Results showed the successful purification of rHA1 using IMAC purification. Minor degradation of the rHA1 was observed in silver stained SDS-PAGE gel, however, the majority of the rHA1 retained its glycosylated state.
Figure 3.11: SDS-PAGE and immunoblotting results of deglycoslated rHA1 protein.

A) Silver stained SDS-PAGE gel, B) The immunoblot using Mouse anti-His (C-Term) antibody; Lane M: ColorPlus™ Prestained Protein Marker; Lane 1: untreated rHA1 protein sample, Lane 2: rHA1 protein underwent native-condition deglycosylation, Lanes 3 to 6: rHA1 protein underwent denaturing-condition deglycosylation with 0.5, 1.0, 1.5 and 2 U of enzyme, respectively. The reaction successfully removed the oligosaccharide chains and resulted in clear protein bands at approximately 45 kDa.
3.3.4 Humoral IgG response in rHA1 vaccinated mice

Mice injected with the vaccine formulations developed no signs of abnormality or change in behavior. Serum samples collected three weeks after the final vaccination were subjected to ELISA for the analysis of HA1-specific IgG responses, and the mice vaccinated with PBS:adjuvant served as negative controls for this assay. The result indicated that mice vaccinated with the rHA1 elicited strong IgG responses (the endpoint titre was 51200, shown in Figure 3.12).

3.3.5 Cell-mediated immune responses in rHA1 vaccinated mice

The T-lymphocyte IL-4 and IFN-γ responses against *P. pastoris* expressed rHA1 were evaluated using splenocytes obtained from immunised mice. In this animal study, only the secretion of IL-4 and IFN-γ were evaluated, which was because the production of other cytokines such as IL-2, IL-6, and IL-10 is not stringently restricted to a single T-lymphocyte sub-population [340]. In the past, the expression of Th1 and Th2 cytokines was considered to be mutually exclusive, however, some T-lymphocytes expressing both Th1 and Th2 cytokines have been observed during differentiation and also in terminally differentiated cells [341-343]. Furthermore, the uniqueness of IL-4 and IFN-γ compare to other Th1 and Th2 cytokines are their positive feedback ability to sustain and proliferate their own T helper lymphocyte sub-population, hence only these two cytokine secretions were evaluated.

Results of the ELISpot assay were expressed as the number of spot-forming T-lymphocytes per $1 \times 10^6$ splenocytes with cells from each mouse assayed in duplicate. In contrast to the control group (PBS), rHA1 immunised mice exhibited significantly elevated IL-4 secretion ($P<0.05$) (Figure 3.13), but no significant stimulation of IFN-γ secretion was observed (Figure 3.14). The Column labeled Con A represents the stimulation with Concanavalin A, a plant-based mitogen known to stimulate T-lymphocyte proliferation and this served as an
internal positive control. In both instances of IL-4 and IFN-γ stimulation, Con A stimulated splenocytes were able to elicit statistically significant T-lymphocyte proliferations from both PBS and rHA1 immunised mice ($P<0.05$). Results were reported as spot count with standard deviation (S.D.).
Figure 3.12: Total IgG responses of immunised mice against *P. pastoris* expressed rHA1.

The endpoint was determined as the dilution at which the optical density (OD) at a wavelength of 450 nm (OD$_{450}$) was three times higher than the background level, which was 51200. This is an aggregated data collected from five mice. The individual IgG response is provided in Appendix 7.
Figure 3.13: ELISPOT assay of IL-4 secreting T-lymphocytes from rHA1 vaccinated mice.

Compared to the control group, splenocytes obtained from rHA1 immunised mice exhibited significant elevation of IL-4 secretion after being stimulated with rHA1. (* $P<0.05$). This is an aggregated data obtained from three mice in the trial. The data of each individual mouse is provided in Appendix 8.
Figure 3.14: ELISpot assay of IFN-γ secreting T-lymphocytes from rHA1 vaccinated mice.

rHA1 stimulated splenocytes were unable to elicit any significant elevation of IFN-γ secretion. However, the internal positive control validated the experiment outcome. This is an aggregated data obtained from three mice in the trial. The data of each individual mouse is provided in Appendix 8.
3.3.6 Virus-neutralising humoral response in rHA1 vaccinated mice

Mice serum samples were subjected to the virus-neutralising hemagglutination inhibition (HI) assay. The HI assay specifically examined the virus-neutralising ability of serum antibodies obtained from rHA1 immunised mice. In contrast to the control group, the serum samples collected from rHA1 immunised mice displayed a significant level of virus-neutralising antibody response ($P<0.05$) (Figure 3.15). The HI titer of serum samples collected from rHA1 immunised mice was determined as $7.4\log_2 \pm 5.5\log_2$. In contrast, serum samples collected from PBS control group demonstrated no virus-neutralising ability.
Figure 3.15: Hemagglutination inhibition assay using serum samples collected from rHA1 immunised mice.

Compared to the control group, mice immunised with rHA1 exhibited significant virus-neutralising antibody responses, at a HI titer of 7.4 in logarithmic base-2 scale. \((P<0.05)\)
3.4 Discussion

In this study, the rHA1 derived from the influenza model strain PR-8 was successfully expressed from *P. pastoris* GS115 via chromosomal integration of recombinant pPICZα-A vector. The rHA1 was collected as a soluble secretory protein in BMMY expression medium after 84 hours of methanol induction, and the rHA1 was subjected to purification, analysis and buffer exchange for later vaccine use.

In a comparison of the silver staining and immunoblotting profiles of the expressed protein samples in different expression medium, BMMY was the medium which gave the best protein expression level; whereas the MMH expression medium was not suitable for rHA1 expression. MMH is minimal methanol medium which contains minimal nutrients without buffering ability, as a result the acidity was increased by accumulation of metabolic byproducts of *P. pastoris*. Hence even the protein expression was induced by the presence of methanol, but the high acidity of the expression medium could inhibit the protein expression or secretion, or even denature the recombinant protein. On the other hand, the BMMH contains the same levels of nutrient as MMH, but the medium was buffered at approximately pH 6.0, and the resulting protein expression was significantly higher than observed in MMH, which indicated the importance of pH buffering in recombinant-protein expression. Moreover, the protein expression was observed highest in the BMMY by estimating the protein concentration on both silver stained SDS-PAGE gel and immunoblotting with PR8 antiserum, and Bradford assay. Therefore, BMMY was selected for further investigations. It is possible that the addition of yeast extract and peptone not only provide extra nutrients for protein expression, but also helps to stabilise secreted protein and prevent or reduce proteolytic activity of secreted proteins [344].
The α-factor secretion signal located on the N-terminus of the rHA1 was able to direct the secretion of recombinant protein across the membrane, and the 6X-His tag located on the C-terminus of the protein was present for purification using Nickel-charged IMAC. After IMAC purification and buffer exchange, the rHA1 appeared to be intact and retains the HA antigenicity with minor protein degradation.

In addition, the rHA1 expressed by \textit{P. pastoris} was glycoslated via the means of post-translational modification. \textit{P. pastoris} has the tendency to glycoslate recombinant glycoprotein by the addition of an average of 8-14 mannose glycol-motif per side-chain [345, 346]. The result of deglycosylation analysis revealed that the highest deglycosylation efficiency was observed with 1 U of the protein deglycosylation enzyme mix for four hours reaction time, as the protein smear was reduced to a minimum; whereas some smearing still appeared right above the protein band and the most was observed in the reaction which underwent non-denaturing deglycosylation. The monoclonal anti-His (C-term) antibody was used for immunoblotting the deglycosylated rHA1 protein, and the resulting bands were clear and with a corresponding molecular weight of approximately 45 kDa.

Glycosylation is a crucial post-translational modification in regards to expression of glycoproteins for recombinant vaccine purposes [347]. The correct glycosylation on synthetic glycoproteins enables the protein to fold and assemble into the native structure, and enhancement of protein stability and secretion processes was also documented [347]. Two forms of glycosylation have been found in \textit{P. pastoris}, the \textit{O}- and \textit{N}-linkage carbohydrates moieties. In lower eukaryotes such as \textit{P. pastoris}, the \textit{O}-linkage glycoslation exists solely with mannose residues; however the function and specificities of these additional \textit{O}-linkage mannose moieties have not yet been fully understood, whereas, the \textit{N}-linked glycosylation in \textit{P. pastoris} is found to be important in human pharmaceutical products. The \textit{N}-linkage
glycosylation in *P. pastoris* involves transferring the lipid-containing oligosaccharide unit “Glc₃Man₉GlcNAc₂” to the recognition protein sequence “Asn-X-Ser/Thr” [292]. In contrast with secretory proteins in *S. cerevisiae*, hyper-glycosylation is often observed as the oligosaccharide chain is elongated in the Golgi via the addition of mannose groups along the outer chain (typically 50 ~ 150 mannose-residues) [345]. These hyper-glycosylated proteins with extended mannose residues raise significant concerns in the application of pharmaceutical products, because the long mannose chains could be hyper-antigenic when administrated intravenously into mammals and these hyper-antigenic proteins will be cleared rapidly via liver [291]. Furthermore, hyper-glycosylation may hinder the antigenic properties or function on the protein of interest by masking important epitopes or functional sites. *S. cerevisiae* glycosylation has terminal α-1,3 linked mannose residues added to expressed proteins, which is known to have hyper-antigenic nature, hence making them unsuitable for therapeutic use [332, 348, 349]. In comparison, the *N*-linkage oligosaccharide chains expressed from *P. pastoris* are more similar to those from higher eukaryotes than in *S. cerevisiae* due to the absence of elongated mannose-residues and terminal α-1,3 linked mannose residues [345, 350, 351]. Therefore, *P. pastoris* is a more preferable host for the expression of recombinant vaccine antigens.

In comparison to the native influenza HA1, Ward *et al.*, 1981 [352] has revealed the composition of the *N*-linked oligosaccharide of influenza HA1 is strain variable. In influenza A/Memphis/102/72 (H3N2) and A/Aichi/2/68 (H3N2), the *N*-linked oligosaccharide compositions are “Fuc₀.₅Gal₁.₅Man₅.₄GlcNac₄” and “Fuc₀.₃Gal₀.₆Man₀GlcNac₂”, respectively. The authors’ result indicated that the oligosaccharide “Glc₃Man₉GlcNAc₂” expressed in *P. pastoris* has similar composition to the native influenza HA1 oligosaccharide at the terminal “-GlcNacₓ” sugar motif. However, the number of mannose-residues in *P. pastoris* expressed *N*-linked oligosaccharide is higher than the native HA1. The influenza HA
glycan-oligosaccharides have been proposed to shield antigenic sites on HA, and promote virus survival. However, addition of glycan-oligosaccharides can also interfere with the receptor binding properties of HA [353]. Therefore, a fine balance must be met regarding the optimal pattern of rHA1 glycosylation to induce protective immunity against the target virus.

In terms of the potential of producing influenza antigens in *P. pastoris*, this study has suggested that such an expression system may be used as an alternative for a more cost-effective and rapid production platform for the development of influenza vaccine. The rHA1 produced in *P. pastoris* can be easily purified from the media facilitated by the presence of 6X-His tag under the native conditions of the protein, and it does not require a costly cell-lysis procedure for protein extraction. In the future, a high protein yield could be accomplished in a large-scale fermentation bioreactor with the help of potent inducible promoters. Safety wise, the recombinant protein expression in *P. pastoris* could be performed in almost serum-free media, which minimises the potential of harbouring harmful pathogens, hence surpassing the safety profile of conventional egg-based influenza vaccines.

The immunogenic potential of *P. pastoris* expressed rHA1 evaluated in the mouse model demonstrated its ability to elicit potent humoral and Th2-biased cell-mediated immune responses. Serum samples collected from rHA1 immunised mice exhibited potent rHA1-specific IgG responses with an endpoint titer of 51200. Furthermore, the HI assay, a “gold-standard” procedure used to evaluate virus-neutralising antibody responses was carried out, and a HI titer of 7.4 log₂ was obtained from serum samples collected from rHA1 immunised mice. This indicates that the rHA1 expressed in *P. pastoris* was able to stimulate the production of HI-specific reactive serum. The elicitation of T-lymphocyte immune responses by rHA1 immunisation was evaluated using the ELISpot assay. The results show that the splenocytes obtained from rHA1 immunised mice had a significant elevation of IL-4.
secretion after rHA1 stimulation but no significant IFN-γ secretion, implying that the elicited T-lymphocyte response was biased towards the CD4+ Th2 proliferation pathway [354, 355].

In Figure 3.13, a large error bar was observed in the rHA1 stimulated splenocytes. As shown in Appendix 8, the amount of IL-4 secretion was different between mice. Such variation might be from a number of factors, including variation in immune responses in different mice, variation in seeding the isolated splenocytes for the assay, and variation of splenocytes in response to stimuli. However, it is not unusual to observe such immunogenic variations in vaccine trials. Furthermore, it has been observed that the amount of IL-4 production in rHA1 stimulated splenocytes was slightly higher than what was observed in Con A stimulated splenocytes. It is possible that the rHA1 stimulated not only Th2 immune responses, but also elicited a strong B cell response. As demonstrated in the animal study, the rHA1-immunised mice exhibited a significant increase of IL-4 and antibody production; hence, it is possible that strong B cell activation was involved in the vaccines immunogenicity. Activated B cells upregulate MHC class II expression, proliferating more B cells and secrete IL-4 [356, 357]. Moller et al., 1986 [358] reported that Con A is not sufficient to induce strong B cell responses through its non-specific mitogenic ability. Therefore, the enhanced B cell activity may have played a role in the slightly higher IL-4 production compare to non-specifically stimulated splenocytes.

Moreover, a small and unique sub-population of T-lymphocytes known as Tc2 might also being involved in this study. In the past, CD8+ T-lymphocytes have been considered as a homogenous population of cytotoxic cells expressing a limited number of cytokines, however, recent studies have revealed that CD8+ T-lymphocytes are similar to their CD4+ counterparts in diversity [359-364]. Similar to the Th1/Th2 terminology, the CD8+ sub-populations were named Tc1 and Tc2. CD8+ T-lymphocytes are differentiated in a similar manner to CD4+
T-lymphocytes, in which, IL-4 and IFN-γ induce the differentiation of naïve CD8\(^+\) T-lymphocytes into Tc\(_2\) and Tc\(_1\), respectively, and IL-12 promotes the proliferation of Tc\(_1\) cells [359, 365, 366]. It has been documented that after differentiation, these effector T-lymphocytes show a stable cytokine production pattern, in which, Tc\(_1\) cells produce IFN-γ and a range of other cytokines but no IL-4; whereas, Tc\(_2\) cells produce IL-4 but no IFN-γ [340].

In conclusion, although Th\(_2\) activation was most likely the main immunological pathway leading to the experiment outcome, however, the possibility of B regulatory cells and Tc\(_2\) cells being involved in the *Pichia*-expressed rHA1 immunogenicity should not be neglected.

The Th\(_2\) proliferation is indicated from the production of cytokines such as IL-4 and IL-5, whereas, Th\(_1\) proliferation is mainly indicated from the production of IFN-γ, IL-2 and TNF-α [354, 355, 367]. In the instances of viral infections such as influenza, CD4\(^+\) Th\(_1\) and Th\(_2\) proliferations are crucial in sustaining CD8\(^+\) T-lymphocyte and humoral responses, respectively [368, 369]. It has been known that subunit vaccines are generally less immunogenic and often require the help from potent adjuvant and repeated vaccinations to elicit long-lasting protective immune responses [41, 57, 60]. As might be expected, the recombinant HA1 vaccine elicited Th\(_2\) responses due to the nature of a subunit vaccine. Furthermore, the prevention of influenza infection mainly relies on a robust Th\(_2\) response to induce the production of neutralising antibodies, which was achieved as shown by the HI assay.

Collectively, the high level production of protective antibody and secretion of IL-4 could be an indication of strong stimulation of B regulatory cells. B cells are the key regulators in both humoral and adaptive immunity. The presence of IL-4 not only induces B cell activation, but also upregulate MHC class II production, and decreases the activation of Th\(_1\) T-lymphocytes [356, 357]. Therefore, the results suggest that the *Pichia*-expressed rHA1 vaccine favours the
activation of Th₂ responses, resulting in strong activation of B cells. These activated B cells produce protective antibodies and IL-4, leading to the upregulation of MHC class II molecule and inhibiting Th₁ responses.

Freund’s adjuvants (FA) were used in the trial to assist rHA1 immunogenicity, where the primary vaccination was adjuvanted with FCA, and two subsequent booster vaccinations were adjuvanted with FIA. The primary vaccination used FCA to stimulate priming immunity, and the subsequent FIA-prepared vaccinations were aimed to boost and maintain a lasting immunity.

FA has been documented to enhance vaccine immunogenicity including rapid uptake of the FA component by dendritic cells, improve phagocytosis, stimulate cytokine production by mononuclear phagocytes, and transient proliferation of CD4⁺ T-lymphocyte responses [370]. FCA contains mycobacterial components which primarily induces CD4⁺ Th₁ response, hence the proliferation of CD8⁺ cell-mediated immune response [371]. FIA on the other hand, contains no PAMP stimuli such as mycobacterial components, hence primarily proliferates CD4⁺ Th₂ responses, and the overall immune responses are more restricted to humoral immunity and the secretion of IL-4 and IL-5 [370, 372].

According to some model studies, the microbial constitutes such as mycobacterial components used in FCA are responsible for the recognition as “non-self” or “danger” via PAMP receptors, resulting in the activation of professional APCs. The activated APCs are promoted to acquire co-stimulatory properties, the “antigen”, and lead to the release of mediators that biases Th₁-type immune responses [372-374]. Nevertheless, under certain conditions, immunisation with FIA could result in the reduction of cell-mediated immune responses. This phenomenon is characterised as the suppression of Th₁ cell-mediated
response by the regulatory function of proliferated Th₂ cells [375-377]. The original interpretation of this phenomenon was a form of immune tolerance. However, further investigations suggested that the failure to stimulate APCs by FIA resulted in a Th₂-directed skewing of the immune response, which favors the development of a strong Th₂-type response [370, 372, 373].

A similar study conducted by Saelens et al., 1999 [235] has examined the immune responses in mice after being vaccinated with the recombinant HA protein of A/Victoria/3/75 (H3N2 subtype) expressed in P. pastoris. Saelens and colleagues expressed HA as soluble secreted molecule in induced culture medium, and the HA protein was purified under native condition by means of an FPLC apparatus equipped with an LCC-501 plus controller and an UV-MII optical unit. Immunised mice exhibited a significant elevation of virus-specific IgG response and complete survival rate in a 10 LD₅₀ challenge using homologous viral strain.

Murugan et al., 2013 [378] reported that the HA protein derived from highly pathogenic (A/Hatay/2004/H5N1) was expressed in the membrane of Pichia. The HA protein was purified under denaturing condition and then refolded prior administered in mice, and HA-specific immune responses were observed in both HI and ELISA assays.

This investigation has demonstrated the immunogenic potential of P. pastoris expressed H1N1 rHA1 for vaccine purposes. The successful IMAC purification under native conditions implied a potential platform for rapid and cost-effective production of vaccine antigen with minimum downstream processes. Furthermore, the antigenicity and immunogenicity of the rHA1 were not compromised due to the purification procedures as the purified rHA1 was able to elicit potent virus-specific antibody and significant T-lymphocyte responses. Therefore, this system may act as an attractive and effective platform for the production of
recombinant HA for the prevention of influenza infections.

A further investigation was carried out by the collaborating institute - Institute of Biotechnology (IBT), Vietnam Academy of Science and Technology. Their study has successfully expressed rHA1 of (A/Hatay/2004/H5N1) as soluble secreted protein, and the rHA1 was purified and the immunogenicity of this protein was evaluated in chickens (personal communication).
Chapter 4
Cloning, Characterisation & Optimisation of the Expression of Influenza HA1 in *Salmonella* vaccine carrier STM1
4.1 Introduction

As previously described and demonstrated, immunisation with rHA1 is capable of eliciting potent virus-specific humoral and CD4+ Th2 cell-mediated immune responses. However, such recombinant vaccine systems require parenteral injection, and the high costs associated with vaccine administration has lead to sub-optimal coverage in the poultry industry [260].

A promising alternative has been discerned from successful demonstrations of in vivo expression of an influenza vaccine antigen delivered by live-attenuated Salmonella vaccine carriers [311, 312]. Salmonella vaccine carriers can be delivered simply via oral delivery through drinking water or aerosol spray. The vaccine is easily produced and able to elicit both humoral immunity, including serum and secretory IgA antibody, and strong cell-mediated immune responses that include cytotoxic and memory T-lymphocytes. Furthermore, recombinant Salmonella not only elicits immune responses against the heterologous antigen upon vaccination, but also to the Salmonella homologous antigens, providing protective immunity against both Salmonella and the heterologous pathogen [98, 379, 380].

In this study, Salmonella Typhimurium strain STM1 (developed at RMIT), harbouring a mutation in the aroA gene region that renders it attenuated, is used as a delivery vector for PR-8 HA1. Different strategies were used to present the antigen from various destinations to optimise immunogenicity. These include one to display the HA1 antigen on the outer membrane of Salmonella (utilising plasmid pHES, encoding the N-terminal signal peptide and the essential C-terminal translocation unit of the ShdA autotransporter derived from S. Typhimurium strain LT-2 [5]), and another to secrete it into the media (pMOhly1, encoding the necessary components of the E. coli α-haemolysin secretion system [6]).

Autotransporters (ATs) are among the largest group of proteins secreted by some pathogenic
Gram-negative bacteria, and consist of an N-terminal signal peptide (SP) followed by a passenger domain, which typically harbours bacterial virulence genes/factors such as cytotoxins, adhesins and proteases [381]. The C-terminal region of ATs fold into a β-barrel that forms a hydrophilic pore and anchors to the outer membrane, which is responsible for the translocation of the passenger domain into the extracellular space [5] (Figure 4.1). The AT vector used in this study, pHES (Figure 4.2), encodes a modified ShdA gene derived from S. Typhimurium. The ShdA is a large outer membrane protein which belongs to the AT family, and has the usual AT β-barrel anchorage domain, and a SP-coupled passenger domain (Figure 4.3). The function of Salmonella ShdA passenger domain is known to be responsible for the attachments to extracellular matrix proteins (i.e. Fibronectin and collagen I) [382, 383].
Figure 4.1: Schematic representation of a native autotransporter.

The native autotransporter consists of the signal peptide, passenger domain, and C-terminal domain consisting of an α-helix and β-barrel which anchors to the bacterial outer membrane (Image adapted from [5]).

Figure 4.2: Map of pHES plasmid.

The ShdA ORF consists of the signal peptide (SP), the passenger domain, followed by the C-terminal anchoring domain α-helix and β-barrel.
Figure 4.3: Tertiary-structure prediction of the *ShdA* C-terminal domain.

The α-helix is represented in red, and the transmembrane amphipathic β-barrel is represented in yellow (Image adapted from [5]).
Another expression vector used in this study was the pMOhly1 (Figure 4.4), which encodes the necessary components of the *E. coli* α-haemolysin secretion system. This vector encodes the *E. coli* α-haemolysin *hly* operon which consists of four genes, *hlyA*, *hlyB*, *hlyC*, *hlyD*, responsible for transporting proteins directly out of the cell [384]. The pMOhly1 vector has the full sequence of the structural genes *hlyC*, *hlyB* and *hlyD* and a truncated version of the *hlyA* gene, *hlyA*’ (Figure 4.5). The full *hlyA* gene is not required for secretion, as only the N-terminus 34 amino acids and the C-terminus 61 amino acids are the regions that are directly transported through the cytoplasm to the extracellular space [322]. pMOhly1 does not require induction for protein expression as it is a constitutive expression vector [322].

Upon protein expression, the first 34 N-terminal amino acids of *hlyA*’ are responsible for assisting the activation of haemolysin system, and the last 61 C-terminal amino acids encode the secretion signal of *hlyA*’ that is responsible for transmembrane exportation of the protein [322]. *HlyC* assists in the synthesis of the constructs and is an acetylase required for post-translational activation of the haemolysin system. Furthermore, the *hlyB* gene is a translocase protein that is located at the C-terminus of the construct and works in conjunction with the *hlyD* protein. *HlyD* is a transmembrane protein which interacts directly with outer membrane (OM) proteins and TLRs, as well as *hlyB* translocase, to form a haemolysin complex which conduits through the cytosol to the extracellular space [384-386] (Figure 4.6).
Figure 4.4: Map of pMOhly1 plasmid.

Figure 4.5: The pMOhly1 hly operon
Figure 4.6: Illustration of the formation of *E. coli* α-haemolysin complex.

The *hlyD* interacts with *hlyB* and Tol-C (TLR) to form a transmembrane complex and allows the recombinant *hlyA* construct to be exported through cytosol to the extracellular space (image adapted from [387]).
The problem of toxicity of over-expressed recombinant protein or the formation of inclusion bodies may be overcame by the use of the plasmid vectors described above. Cytoplasmic accumulation of recombinant protein often results in inactive and insoluble products [388]. The signal peptide encoded in pHES plasmid prevents cytoplasmic accumulation of the fusion rHA1 in STM1 by translocating the protein through the periplasmic space to bacterial outer membrane. pMOhly1 plasmid encodes the necessary components of the *E. coli* α-haemolysin secretion system, which secrete the fusion rHA1 out of the cell.

The aim of work described this chapter was as follows:

- To obtain the HA1 DNA sequence from viable PR-8 virus using RNA extraction, reverse transcription and PCR amplification.
- To clone the amplified HA1 DNA sequence into pHES and pMOhly1 plasmids, and transform these plasmid constructs into *Salmonella* STM1.
- To optimise the rHA1 protein expression from these vaccine constructs.
- To evaluate and characterise the ability of pHES surface-display and pMOhly1 extracellular secretion.
4.2 Materials and methods

4.2.1 Overview

The PR-8 virus HA1 gene used in this study was directly obtained from viable PR-8 virus. The PR-8 virus was cultivated in a mammalian cell-line, and the DNA sequence encoding the HA1 gene was firstly obtained by total RNA isolation, followed by reverse transcription into cDNA. The gene of interest was then amplified by PCR, using designated primer pairs listed in Table 2.6.

For the construction of HA1 fused pHES vector (pHES/HA1), the PR-8 HA1 gene was inserted in the passenger domain of the encoded ShdA sequence, and the construct was transferred into Salmonella STM1 for surface display of the antigen (Figure 4.7). In addition, the pHES vector consists a 6x-His tag located close to the SP of the passenger domain and a lac^Iq-Plac promoter. This promoter not only has the normal function of IPTG inducible protein expression, but the expression also can be inhibited via the presence of glucose.

For the construction of HA1 fused pMOhly1 vector (pMOhly/HA1), the amplified PR-8 HA1 gene was inserted in the middle of the truncated hlyA' gene sequence (Figure 4.8). The signal peptide encoded in the hlyA' directs the fusion rHA1 out of the cell through E. coli α-haemolysin complex (as shown in Figure 4.6)
Figure 4.7: Development of pHES/HA1 plasmid construct.

A) Schematic representation of the ShdA operon encoded in pHES plasmid with the inserted influenza HA1 (Image modified from [5]). B) Map of the complete pHES/HA1 construct. The HA1 gene was inserted in the passenger domain (PD) for surface display.
Figure 4.8: Development of pMOhly1/HA1 plasmid construct.

A) Schematic representation of the *hly* operon encoded in pMOhly1 plasmid with the inserted influenza HA1. B) Map of the complete pMOhly1/HA1 construct.
4.2.2 Preparation of HA1 gene from PR-8 virus

4.2.2.1 Virus cultivation and purification

MDCK cell monolayers were prepared as described in Section 2.8, and the method for PR-8 virus propagation and purification is described in Section 2.9.

4.2.2.2 RNA extraction and cDNA synthesis

Purified PR-8 virus was subjected to RNA extraction and cDNA synthesis as described in Section 2.4.

4.2.2.3 PCR amplification using cDNA template

The primer pairs used for these amplifications are listed in Table 2.6. For the amplification of pMOhly1 insert, the primer pairs were pMOhly1_SbfI_For and pMOhly1_SbfI_Rev. For the pHES insert, the pairs were pHES_EcoRI_For and pHES_PvuI_Rev.

The general PCR procedure is described in Section 2.6.2.2. However, optimisation of the PCR reactions was required. Briefly, the cDNA concentration was estimated using a Microplate luminometer (BMG LABTECH GmbH, Germany), and the PCR reactions were performed using different amount of cDNA template (between 10-100 ng), for each primer pair. Furthermore, the annealing temperatures for these reactions were lowered to 48°C for both primer pairs.

4.2.2.4 TA cloning strategy

Having successfully amplified both HA1 DNA inserts, the PCR products were cloned directly into the TA cloning vector pCR2.1 according to the manufacturer’s instructions (Invitrogen, USA). The ligation products were cleaned using the QIAquick gel extraction kit (Section 2.5.2). The purified ligation products were used for electroporation (Section 2.6.5), into electrocompetent E. coli DH5α (Section 2.6.4).
Following transformation, the cells were cultured on LB agar (containing 40 μg/mL X-Gal, 1 mM IPTG and 100 μg/mL Ampicillin), for Blue/White screening. These putative clones were then grown in LB broth containing 100 μg/mL Ampicillin. Clones were screened by restriction enzyme digestion. Positive clones were grown overnight and stored at -80°C until use.

4.2.3 Sub-cloning

4.2.3.1 Background development of constructs

The primer pairs used for cloning in this study (Table 2.6) were designed based on the HA gene sequence of influenza PR-8 virus obtained from GenBank (access number: EF467821).

For the construction of pHES/HA1, the HA1 gene sequence was designed to be inserted into the multiple cloning site of the pHES vector immediately downstream of the 6x-His tag sequence. The forward and reverse primers for pHES/HA1 cloning were incorporated with EcoRI and PvuI (New England Biolabs, USA), enzyme sites respectively, and a T nucleotide was placed immediate upstream of the PvuI enzyme site for in-frame protein expression. These restriction sites were used as they did not appear within the PR-8 HA1 gene.

For the construction of pMOhly1/HA1, the presence of an NsiI site in the PR-8 HA1 sequence precluded its use in the primers designed for cloning into pMOhly1. The SbfI enzyme site was not present in the PR-8 HA1 sequence, and the recognition sites of NsiI (ATGCA↓T) and SbfI (GG↑ACGTCC) have complementary overhangs of ACGT for ligation, therefore the pMOhly1 forward and reverse primers were incorporated with SbfI sites for subsequent cloning. A sequence of “GTGATGGTGATGGTGATG” encoding 6x-His was placed immediately upstream of the reverse primer SbfI site for the incorporation of a 6x-His tag into the pMOhly1/HA1 protein. Additional CT nucleotide bases were placed in front of the 6x-His sequence for in-frame expression of the 6x-His tag.
4.2.3.2 Preparation of pHES and pMOhly1 vectors

For pMOhly1-bearing *E. coli*, the cells were grown overnight in LB broth containing 100 μg/mL Ampicillin, whilst pHES-bearing *E. coli* was grown overnight in LB broth containing 40 μg/mL Chloramphenicol and 2% (w/v) glucose. Following incubation, pHES and pMOhly1 plasmids were isolated from overnight cultures using the QIAprep® spin miniprep kit (described in Section 2.5.1).

The pHES vector was digested with *Pvu*I and *Eco*RI for four hours in a 37°C water bath; whereas the pMOhly1 vector was digested with *Nsi*I for two hours under the same conditions.

Following digestion, the digested vectors were run on a 0.5% agarose gel and the appropriate fragments were excised from the gel and purified using the QIAquick gel extraction kit (as described in Section 2.5.2). The purified DNA fragments were used immediately for ligation.

4.2.3.3 Sub-cloning HA1 insert into pHES vector

The pCR2.1 clone harbouring HA1 insert for pHES/HA1 was grown and the plasmid isolated via QIAprep® spin miniprep kit. The PCR fragment was then digested from pCR2.1 using *Eco*RI and *Pvu*I, and run on a 0.5% agarose gel. The appropriate fragment was excised from the gel and purified using the QIAquick gel extraction kit. The fragment was then directly ligated into the previously digested pHES vector (Section 4.2.3.2). Following ligation, the ligation product was purified (as described in Section 2.5.2), and 4 μL of ligation product was used for electroporation into electrocompetent *E. coli* DH5-α. The cells were then grown on LB agar (containing 40 μg/mL chloramphenicol and 2% (w/v) glucose), and incubated for 16 hours at 37°C. Putative clones were sub-cultured onto fresh LB agar for screening.
4.2.3.4 Sub-cloning HA1 insert into pMOhly1 vector

Similar to procedures used in Section 4.2.3.3. The restriction enzyme was replaced with SbfI, and cells grown on LB agar (containing 100 μg/mL Ampicillin).

4.2.4 Screening of clones

The putative clones were grown in LB broth (containing appropriate supplements), overnight at 37°C with 200 rpm shaking, and plasmid were isolated using the QIAprep® spin miniprep kit.

For pHES clones, the clone was confirmed using restriction enzyme digestion (EcoRI and PvuI), and PCR amplification using combinations of primers listed in Table 4.1. The annealing temperature was set to 48°C, and the remaining parameters were as described in Section 2.6.2.2.

Screening for pMOhly1 clones was solely based on PCR amplification, as the NsiI and SbfI enzyme sites are not recleavable following ligation. Colony PCR amplification (Section 2.6.2.3) using the following combinations of primers (Table 4.2) was used for the confirmation of HA1 insertion and appropriate orientation.
Table 4.1: Screening PCR primer combinations for pHES/HA1 clone.

<table>
<thead>
<tr>
<th>Primer combination</th>
<th>Expected fragment sizes being amplified (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEQF_pHES + SEQR_pHES</td>
<td>1620</td>
</tr>
<tr>
<td>SEQF_pHES + SEQR_PR8HA1</td>
<td>867</td>
</tr>
<tr>
<td>SEQR_pHES + SEQF_PR8HA1</td>
<td>589</td>
</tr>
</tbody>
</table>

Table 4.2: Screening PCR primer combinations for pMOhly1/HA1 clone, and for confirmation of insert orientation.

<table>
<thead>
<tr>
<th>Primer combination</th>
<th>Expected fragment sizes being amplified (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correct insert orientation</td>
<td></td>
</tr>
<tr>
<td>SEQF_pMOhly1 + SEQR_pMOhly1</td>
<td>1618</td>
</tr>
<tr>
<td>SEQF_pMOhly1 + SEQR_PR8HA1</td>
<td>903</td>
</tr>
<tr>
<td>SEQR_pMOhly1 + SEQF_PR8HA1</td>
<td>551</td>
</tr>
<tr>
<td>Incorrect insert orientation</td>
<td></td>
</tr>
<tr>
<td>SEQF_pMOhly1 + SEQR_pMOhly1</td>
<td>1618</td>
</tr>
<tr>
<td>SEQF_pMOhly1 + SEQR_PR8HA1</td>
<td>No product</td>
</tr>
<tr>
<td>SEQR_pMOhly1 + SEQF_PR8HA1</td>
<td>No product</td>
</tr>
</tbody>
</table>
4.2.5 DNA sequence analysis for confirmation of clones

The putative clones selected as previously described were subjected to DNA sequence analysis (Section 2.6.6). The construct plasmids were isolated using QIAprep® spin miniprep kit, and the DNA concentration was estimated using Microplate luminometer (BMG LABTECH GmBH, Germany). Each isolated plasmid was prepared with four primers (as listed in Table 2.6), and the individual DNA sequences were amplified and analysed.

4.2.6 Passage of plasmids

Having successfully cloned PR-8 HA1 into each expression vector, and confirmed by sequence analysis, the plasmid constructs were isolated from E. coli DH5-α using the QIAprep® spin miniprep kit. Electrotransformation (Section 2.6.5) was used to transform the plasmid into the intermediate vector strain, S. Typhimurium LT2-9121. The plasmid constructs were then isolated a second time using the same method described above from the S. Typhimurium LT2-9121 and electrotransformation was used again to incorporate these passaged plasmids into the S. Typhimurium STM1 vaccine strain. The STM1 harbouring pHES/HA1 and pMOhly1/HA1 will be referred as pHES/HA1/STM1 and pMOhly1/HA1/STM1 hereafter. Negative control clones were also generated by passage of blank pHES and pMOhly1 vectors into STM1, which will be referred as pHES/STM1 and pMOhly1/STM1, respectively.
4.3 Materials and methods for protein expression, and characterisation

4.3.1 pHES/HA1/STM1 protein expression

4.3.1.1 Pilot expression of rHA1 protein

The pHES/HA1/STM1 clone was grown in 10 mL LB broth containing 40 μg/mL chloramphenicol and 2% (w/v) glucose overnight at 30°C with 100 rpm orbital shaking. The next day, overnight cultures were diluted to a final optical density at 600 nm (OD\textsubscript{600nm}) of 0.5 in fresh LB broth containing 0.5 mM IPTG and 40 μg/mL chloramphenicol, in a total volume of 200 mL, and the culture was incubated for five hours at 37°C with 200 rpm orbital shaking. Ten milliliter samples were collected prior to induction with IPTG and every hour thereafter. Samples were washed twice with ice-cold 10 mM Tris-HCl pH 8.0, and resuspended using the same buffer (supplemented with Protease Inhibitor Cocktail (Cell Signalling Technology, USA)), followed by cell lysis using sonication (Section 2.7.1). Protein concentration of the supernatant was measured using the Bradford assay (Section 2.7.2), and up to 30 μL of lysed cell supernatant was analysed using SDS-PAGE and immunoblotting (Section 2.7.3 and Section 2.7.4).

4.3.1.2 Optimisation of rHA1 expression

Based on the same parameters used in Section 4.3.1.1, different IPTG concentrations (0.1 mM, 0.5 mM and 1.0 mM), were used in the expression media for optimising protein expression levels. Different expression media were also used for optimising protein expression levels, such as BHI, LB, MH, and NB (as listed in Section 2.2.5.1). An additional step was included to ensure the promoter-inhibitory agent – glucose was removed from the expression media, in which, the overnight grown pHES/HA1/STM1 was washed three times with sterile PBS prior to inoculation into expression media. Different temperature conditions
were used to optimise the rHA1 protein yield; including 30\(^0\)C for overnight expression and 34\(^0\)C for 10 hours expression. Samples were collected, processed and analysed as described in Section 4.3.1.1.

4.3.1.3 Cell membrane fractionation

Having optimised the protein expression conditions, the rHA1 protein was analysed using cell fractionation, protocol adapted from [5]. A sample of the expressed culture was harvested at the time-point where the highest rHA1 protein concentration was observed. The pHES/HA1/STM1 cells were washed twice with 10 mM Tris-HCl pH 8.0, and resuspended in the same buffer supplemented with Protease Inhibitor Cocktail (PIC), and then sonicated as described in Section 2.7.1. Following sonication, the supernatant was passed through a 0.2 μm filter, and 8 mL of protein extract was subjected to centrifugation at 100,000 x g for one hour at 4\(^\circ\)C. After ultracentrifugation, the supernatant was collected and stored at -20\(^\circ\)C, and the pellet was resuspended in 10 mM Tris-HCl pH 8.0 with supplements of PIC and 5% (v/v) Triton X-100 (Sigma-Aldrich). The sample was incubated on ice for 30 minutes and centrifuged again at 100,000 x g for one hour at 4\(^\circ\)C. The supernatant was collected and stored at -20\(^\circ\)C, and the pellet was resuspended in 10 mM Tris-HCl pH 8.0 with supplements of PIC and 4 M Urea (Sigma-Aldrich, USA). The sample was subjected to another cycle of ice incubation and ultracentrifugation, and the supernatant was collected and stored at -20\(^\circ\)C. The remaining pellet was resuspended in 10 mM Tris-HCl pH 8.0 with PIC and stored at -20\(^\circ\)C. The protein concentrations of the collected protein fractions were measured using the Bradford assay, and 30 μg of fraction samples were evaluated using SDS-PAGE and immunoblotting.

4.3.1.4 SDS-PAGE and immunoblotting

For pilot and optimisation studies of pHES/HA1/STM1 protein expression, the SDS-PAGE was stained using EzStain AQUA (ATTO, Co., USA) as per the manufacturer’s instructions.
As for immunoblotting (Section 2.7.4), the membranes were probed with either primary 6x-His antibody or PR-8 antiserum, and coupled with conjugate secondary antibodies, anti-mouse IgG AP and anti-rabbit IgG AP, respectively (Section 2.2.5.4). To develop AP-conjugated antibodies, BCIP/NBT solution (Sigma-Aldrich, USA) was added for incubation with gentle agitation. The reaction was stopped by washing with dH₂O.

### 4.3.2 pMOhly1/HA1/STM1 protein expression

#### 4.3.2.1 Expression of extracellular rHA1 from pMOhly1/HA1/STM1

pMOhly1/HA1/STM1 was cultured in 10 mL of BHI supplemented with 100 μg/mL ampicillin for 18 hours at 37°C with 150 rpm orbital shaking. Following incubation, the culture was centrifuged at 5,450 x g for 10 minutes at 4°C, and the supernatant was collected and passed through a 0.45 μm filter. The supernatant was then subjected to Nickel-charged IMAC purification.

#### 4.3.2.2 IMAC purification for detection of extracellular fusion rHA1

The same protocol was used as described in Section 3.2.3. Briefly, 10 mL of supernatant prepared from overnight culture was added to 3 mL of chelating Sepharose fast flow® resin, which was previously charged with Nickel ions and equilibrated with Native Binding Buffer. The column was incubated for one hour at 4°C on a roller mixer (Ratek, Australia). The column was placed upright, and the sample allowed flow through. Fifteen CV of Native Wash Buffer was loaded, followed by the addition of 5 CV of Native Elution Buffer. The elution fractions were collected in 1 mL aliquots and stored at -20°C until analysis.
4.3.2.3 SDS-PAGE and Immunoblotting

The elution fractions collected from IMAC purification were examined using silver stained SDS-PAGE (described in Section 2.7.3.2) and Immunoblotting with PR-8 antiserum (described in Section 2.7.4).

4.4 Results

4.4.1 Tissue culture, virus propagation and gene amplification

Healthy MDCK cells were cultivated and successfully maintained as an adherent cell-line. Upon introduction of PR-8 virus, the cytopathic effect of cell death could be observed between two to four days post-infection (Figure 4.9).

4.4.2 Virus purification and HA titre

PR-8 virus was successfully extracted and purified, and an HA test (Section 3.2.9.3.1) was carried out to determine the HA titre of the purified samples. The results indicated an average between 400-800 HA units can be obtained from the procedure described in Section 2.9, using two flasks (75 cm²) of 100% confluent MCDK monolayer, which was infected with 1:1000 diluted virus stock (originally 400 HA units), and harvested when more than 50% of MDCK cells detached from the bottom of the flask.
Figure 4.9: Photographic images of healthy and PR-8 virus infected MDCK cells under 40X magnification.

A) healthy MDCK cell-line about 50% confluency; B) 4 days post-infection of a 100% confluent monolayer; C) negative control flask of image B), where no virus was introduced, but media replaced with virus propagation media (Section 2.9.2).
4.4.3 RNA extraction and cDNA synthesis

Two hundred microlitres of freshly purified PR-8 virus was subjected to RNA extraction using TRIzol® LB reagent (Section 2.4.1). The resulting RNA pellet was resuspended in 20 μL of chilled DNase/RNase-free water, and immediately used for cDNA synthesis. The concentration of extracted viral RNA was estimated using a spectrophotometer at 260 nm, and the results indicated the sample contained approximately 3.0 μg/μL of RNA. An equivalent to 6 μg of extracted RNA molecule was used as a template for cDNA synthesis, and approximately 1.5 μg/μL cDNA was generated from the reaction. A 1% agarose gel was prepared to visualise the RNA and cDNA samples (Figure 4.10).
Figure 4.10: RNA extraction and cDNA synthesis.

Lane M: *PstI*/λ-DNA marker, Lane 1: extracted PR-8 RNA (3.0 μg), Lane 3: complementary cDNA reverse transcribed from PR-8 RNA extract (3.0 μg). No clear bands were observed. These smearings are considered normal for separating RNA or single-strand DNA molecules on standard gel electrophoresis.
4.4.4 Amplification of the influenza HA1 gene

The initial PCR amplification of the HA1 gene used 0.5 μg, 1.0 μg and 5.0 μg of cDNA molecules as a template, and annealing temperature at 55°C did not result in any PCR products (data not shown). The PCR annealing temperature was lowered to 50°C and a DNA fragment at the expected size of 0.98 kbp was amplified using pHES_EcoRI_For and pHES_PvuI_Rev primer pair with 1.0 μg of cDNA template, but no DNA product was amplified using pMOhly1_SbfI_For and pMOhly1_SbfI_Rev primer pair (Figure 4.11).

The PCR reaction was repeated again using a 48°C annealing temperature, resulting in DNA fragments at the expected size of 0.98 kbp using pHES_EcoRI_For and pHES_PvuI_Rev primer pair with 0.5 μg, 1.0 μg and 5.0 μg of cDNA molecules as template. On the other hand, PCR reactions with pMOhly1_SbfI_For and pMOhly1_SbfI_Rev primer pair only resulted in one expected DNA fragment at the expected size of 0.98 kbp in the reaction which was supplied with 1.0 μg of cDNA template (Figure 4.12).

The freshly amplified HA1 gene was immediately subjected to TA cloning.
Figure 4.11: PCR amplification of influenza HA1 gene at annealing temperature of 50°C.

Lane M: *PstI/λ*-DNA marker, Lane 1, 2 and 3: PCR reactions using pHES primer pair with 0.5 μg, 1.0 μg and 5.0 μg of cDNA template, respectively. Lane 4, 5, and 6: PCR reactions using pMOhly1 primer pair with 0.5 μg, 1.0 μg and 5.0 μg of cDNA template, respectively. The PCR reactions used 55°C as annealing temperature did not result in any PCR product; whereas, by lowering down the annealing temperature to 50°C, an expected DNA fragment of approximately 0.98 kbp was amplified in the reaction with pHES primer pair with 1.0 μg cDNA template.
Figure 4.12: PCR amplification of influenza HA1 gene at annealing temperature of 48°C.

Lane M: PstI/λ-DNA marker, Lane 1: 1.0 μg of freshly synthesised influenza cDNA, Lane 2, 3 and 4: PCR reactions using pHES primer pair with 0.5 μg, 1.0 μg and 5.0 μg of cDNA template, respectively. Lane 5, 6, and 7: PCR reactions using pMOhly1 primer pair with 0.5 μg, 1.0 μg and 5.0 μg of cDNA template, respectively. All three reactions using pHES primer pair generated expected DNA fragments at a size of approximately 0.98 kbp, and similar fragment was amplified in the reaction supplied with pMOhly1 primer pair and 1.0 μg of cDNA template.
4.4.5 TA cloning strategy

Both pHES and pMOhly1 inserts were ligated into the pCR2.1 vector, and transformed into electrocompetent *E. coli* DH5-α cells via electroporation, and then inoculated onto agar containing X-gal for Blue/White screening (described in Section 4.2.2.4).

Following overnight incubation at 37°C, three white colonies were collected from each pHES and pMOhly1 insert group for screening. For suspected clones harbouring pHES insert, the plasmids were digested with *EcoR*I and *Pvu*I; whereas, for suspected clones harbouring pMOhly1 insert, the plasmid were digested with *SbeI* alone. The digested plasmids were run on a 1% agarose gel for examination (Figure 4.13).

The result indicated that pHES insert was successfully ligated into clone No. 1 and No. 3 from the pHES group, and pMOhly1 insert was successfully ligated into clone No. 3 of the pMOhly1 group. Clone No. 1 of the pHES group and clone No. 3 of the pMOhly1 group were chosen for subsequent use.
Figure 4.13: Screening for pCR2.1 vectors harbouring pHES or pMOhly1 insert.

Lane M: *PstI*/λ-DNA marker, Lane 1: digested pHES insert, Lane 2, 3 and 4: enzymatic digested pCR2.1 clones harbouring pHES insert, Lane 5: digested pMOhly1 insert, Lane 6, 7 and 8: enzymatic digested pCR2.1 clones harbouring pMOhly1 insert. Partial digestion was evident in Lanes 6, 7 and 8. pHES clone from Lane 2 and pMOhly1 clone from Lane 8 were chosen for subsequent use.
4.4.6 Cloning and construction of pHES/HA1 and pMOhly1/HA1 in E. coli DH5-α

The inserts were digested from pCR2.1 using the corresponding restriction enzymes (pHES insert: *Pvu*I/*EcoRI*), pMOhly1 insert: *SbfI*). The pHES insert was excised from an agarose gel and ligated into the *Pvu*I/*EcoRI* digested pHES vector using T4 DNA ligase. The pMOhly1 insert was extracted and ligated into the *Nsi*I digested pMOhly1 vector using the same ligase. The resulting plasmids were named pHES/HA1 and pMOhly1/HA1, respectively. Resulting clones were screened for successful insertion of the influenza HA1 gene, and directionality of the insert for pMOhly1/HA1.

4.4.6.1 Screening and confirmation for pHES/HA1 clone

Plasmids were extracted from suspected pHES clones, and digested using *Pvu*I and *EcoRI* for screening (Figure 4.14). The result indicated the ligation frequency was quite low and many of the colonies screened contained the empty vector only. One clone (No. 36), was shown to have a digested DNA fragment of an expected size of 0.98 kbp matching to the size of digested pHES insert, but the fragment matching to digested pHES vector was very faint, hence the clone was cultured and plasmid extracted for re-examination. The digested pHES clone produced two DNA fragments at the expected sizes of 4.5 and 0.98 kbp matching the sizes of digested pHES vector and insert, respectively (Figure 4.15).

A PCR reaction using an annealing temperature of 48°C coupled with three combinations of primers (listed in Table 4.1), was used to confirm the clone. Three strong bands at the expected sizes of 1.620, 0.867 and 0.589 kbp (Figure 4.16), were observed, which indicated the successful insertion of pHES insert into the vector.
Figure 4.14: Screening for pHES/HA1 clones.

Lane M: *PstI/-*DNA marker, Lane 1: digested pHES vector, Lane 2: digested pHES insert, Lane 3 to 18: clones subjected to screening. Clone No. 36 (Lane 15) appeared to have the expected DNA fragments, hence it was subjected to confirmation.
Figure 4.15: Confirmation of pHES/HA1 clone.

Enzymatic digestion of pHES/HA1 using EcoRI and PvuI. Lane M: PstI/λ-DNA marker, Lane 1: digested pHES vector, Lane 2: digested pHES insert, Lane 3: digested pHES construct.
Figure 4.16: PCR confirmation of pHES/HA1 construct using three combinations of primers (refer to Table 4.1 for each reaction).

Lane M: PstI/λ-DNA marker, Lane 1: primer combination (SEQF_pHES + SEQR_pHES), Lane 2: primer combination (SEQF_pHES + SEQR_PR8HA1), Lane 3: primer combination (SEQR_pHES + SEQF_PR8HA1). The PCR reactions confirmed the successful ligation of influenza HA1 gene into pHES vector in the correct orientation.
4.4.6.2 Screening and confirmation for pMOhly1/HA1 clone

Colony PCR using an annealing temperature of $48^\circ$C, and coupled with SEQF_pMOhly1 + SEQR_pMOhly1 primer pair (listed in Table 4.2), was used for screening pMOhly1 clones. The ligation frequency was relatively low compared to the construction of pHES clone, as hundreds of colonies contained only the empty pMOhly1 vector. Two pMOhly1 clones (clone No. 290 and No. 495), were shown to have PCR product at the expected size of approximately 1.62 kbp (Figure 4.17). The putative clones were screened for directionality using the primer combinations listed in Table 4.2. For Clone No. 290, three products at the expected sizes of 1.62, 0.90 and 0.55 kbp were amplified, which indicated the successful ligation of influenza HA1 gene into pMOhly1 hlyA in the correct orientation (Figure 4.18). The PCR results of Clone No. 495 indicated that influenza HA1 gene was ligated into pMOhly1 vector in the incorrect orientation.
Figure 4.17: PCR screening for putative pMOhly1/HA1 clones using primer combination SEQF_pMOhly1 + SEQR_pMOhly1.

Lane M: PstI/λ-DNA marker, Lane 1 to 9: putative pMOhly1/HA1 clones. The PCR result shows a strong band at an expected size of 1.62 kbp from Clone No. 290 (lane 9). This PCR product indicated an extra 0.98 kbp was inserted in the middle of pMOhly1 hlyA Open Reading Frame, which suggested the successful insertion of the influenza HA1 gene. Other putative clones have only shown the amplification of the hlyA gene alone, or no PCR product at all. Clone No. 290 was then screened using the combinations of primer listed in Table 4.2 for directionality of the insert.
Figure 4.18: Directional screening of putative pMOhly1/HA1 clones (primer combinations refer to Table 4.2).

Three PCR reactions were set up for each clone according to the primer combinations listed in Table 4.4. Ticks indicate a PCR product is expected if the insert is in the correct orientation, crosses indicate a PCR product is not generated as the insert is in the incorrect orientation. From the results, Clone No. 290 appeared to have the influenza HA1 gene inserted into pMOhly1 hylA ORF in the correct orientation.
4.4.6.3 Sequencing analysis for the confirmation of pHES/HA1 and pMOhly1/HA1 clone

Sequencing of these clones revealed that the PCR-confirmed pHES/HA1 clone and pMOhly1/HA1 clone had the correct insert in the correct orientation. In comparison with the sequence information of influenza A PR-8 HA gene, two point substitutions were observed in the HA1 inserts in both clones at the same position. The result suggests that the mutations could not have being introduced during the cloning procedure, but had always existed in the virus stock (Figure 4.19). In silico analysis confirmed that one of the base substitutions will result in an amino acid substitution (Figure 4.20A & Figure 4.20B). BLAST analysis shows that the substituted amino acid (Serine) at this position does occur in other strains of influenza virus.

4.4.7 Transformation of the plasmid constructs into vaccine strain STM1

The plasmids of pHES/HA1 and pMOhly1/HA1 were passed through the intermediate strain of *S. Typhimurium* LT2-9121 in STM1 so that transformation would occur at a higher frequency between the two related bacterial strains. These vaccine strains will be referred to as pHES/HA1/STM1 and pMOhly1/HA1/STM1. The STM1 transformants were confirmed using colony PCR reactions coupled with combinations of primers listed in Table 4.1 and Table 4.2 (Figure 4.21). Original pHES and pMOhly1 plasmids were also transformed into STM1 as negative control strains for subsequent analysis.
Figure 4.19: Illustration of two base-substitutions found in the virus stock.

The HA1 DNA sequence of influenza PR-8 virus. The base-substitutions were coloured in red.

These two DNA bases were Cytosine (C), and they were substituted with Thymine (T).
Figure 4.20: Amino acid substitution of the rHA1 based on the base substitutions.

A) Alignment of the reference pHES/rHA1 protein sequence and the amino acid substituted pHES/rHA1 protein. B) Alignment of the reference pMOhly1/rHA1 protein and the amino acid substituted pMOhly1/rHA1 protein. These alignments have indicated that the DNA base-substitutions found in the sequence analysis will result in an amino acid substitution in both pHES/rHA1 and pMOhly1/rHA1 fusion proteins, in which, a Proline (P) will be substituted with Serine (S) in the rHA1 proteins.
Figure 4.21: PCR confirmation of STM1 transformants.

A) pHES/HA1 transformants amplified using primer combinations listed in Table 4.1.

B) pMOhly1/HA1 transformants amplified using primer combinations listed in Table 4.2. The plasmid constructs were successfully transformed into vaccine strain STM1.
4.4.8 pHES/HA1/STM1 protein expression, optimisation and characterisation

The pilot expression described in Section 4.3.1.1 did not result in any detectible rHA1 from cell lysates. Several detailed steps were carefully considered to increase the rHA1 protein yield and enhance the assay sensitivity. An extra step was included to ensure glucose – the promoter-inhibitory agent was reduced to a negligible level by washing the overnight cells three times with sterile ice-cold PBS, and resuspending the cell pellet with expression medium to an OD_{600nm} of 0.5, in a total volume of 200 mL. The amount of protein loaded onto SDS-PAGE gel for subsequent immunoblotting was increased to approximately 100 μg per well. These changes enabled the detection of a protein at approximately 80 kDa, which is similar to the predicted MW of rHA1 expressed in pHES plasmid at 79.26 kDa (prediction was performed using the ExPASy Compute pl/Mw tool) (Figure 4.22).

The expression was then optimised by varying the amount of inducing agent, IPTG. The clones were expressed in LB broth supplemented with 0.1 mM, 0.5 mM and 1.0 mM of IPTG. The result indicated that the addition of 1.0 mM of IPTG allowed optimal yield of rHA1 protein expression at approximately three hours post-induction (Figure 4.23). A time-course study of the protein expression was performed to determine the optimal time for harvesting rHA1. Results indicated that the highest rHA1 protein yield was reached three hours post-induction, and the yield gradually decreased thereafter (Figure 4.24). Different culture media were also tested for the optimal rHA1 yield, which included BHI, MH, LB and NB broth. The results showed that LB broth enabled the optimal rHA1 yield, followed by MH broth (Figure 4.25). BHI and NB broth were unable to support the expression of rHA1 from pHES/HA1/STM1.
Figure 4.22: Pilot expression of rHA1 from pHES/HA1/STM1.

Pilot expression: Western blot probed using 6x-His antibody, M: Precision Plus Protein™ Dual Color Standards, Number (1-5) above blot indicate the number of hours post-induction. Three faint protein bands with MW at approximately 80 kDa were observed in lane 3 to 5, indicating the expression of pHES/HA1 fusion protein. Three additional protein bands were also observed at approximately 30 kDa, which remained detectible in Western blot probed with 6x-His antibody, but not reactive with PR-8 antiserum (in the subsequent Western blot images). This indicated that these 30 kDa protein could not have being the breakdown product of pHES/HA1 fusion protein. It is possible that these proteins are the stress related proteins, which have been expressed due to the expression of the pHES/HA1 fusion protein [389].
Figure 4.23: Optimisation of pHES/HA1/STM1 expression by media supplemented with different concentrations of IPTG.

A) The immunoblot using 6x-His antibody, B) The immunoblot using PR-8 antiserum; Lane M: Precision Plus Protein™ Dual Color Standards, Lane 1: pHES/HA1/STM1 at 0 hour, Lane 2 and 3: pHES/HA1/STM1 induced with 0.1 mM IPTG for 3 and 6 hours, Lane 4 and 5: pHES/HA1/STM1 induced with 0.5 mM IPTG for 3 and 6 hours, Lane 6 and 7: pHES/HA1/STM1 induced with 1.0 mM IPTG for 3 and 6 hours. Optimal rHA1 protein yield was achieved in the media induced with 1.0 mM IPTG for three hours.
Figure 4.24: Time-course study of rHA1 protein expression using media supplemented with 1.0 mM IPTG.

A) The immunoblot using 6x-His antibody, B) The immunoblot using PR-8 antiserum; Lane 1: pHES/STM1 clone 5 hours post-induction, Lane M: Precision Plus Protein™ Dual Color Standards, Lane 2 to 7: pHES/HA1/STM1 clone 0 to 5 hours post-induction. Results indicate that rHA1 protein yield increases gradually after induction and reached the maximum yield at approximately three hours post-induction, and yield decreases thereafter.
Figure 4.25: Optimisation of pHES/HA1/STM1 expression using different media.

A) The immunoblot using 6X-His antibody, B) The immunoblot using PR-8 antiserum; Lane M: Precision Plus Protein™ Dual Color Standards, Lane 1: pHES/STM1 clone at 5 hours post-induction, Lane 2 to 5: pHES/HA1/STM1 clone expressed in BHI, LB, MH and NB broth, respectively. Results indicate that BHI and NB broth were unable to support the expression of rHA1, and LB broth appears to provide the optimal expression conditions for the clone.
Based on the optimised parameters obtained, expression of rHA1 at a lower temperature was also investigated. However, the results indicated that 37\(^\circ\)C was still the optimal temperature for the expression of rHA1. Overnight expression of rHA1 at 30\(^\circ\)C did not result in any detectable rHA1 using immunoblotting, and 34\(^\circ\)C expression resulted in a similar pattern of rHA1 expression to the profile observed from 37\(^\circ\)C expression (data not shown). In conclusion, the rHA1 was successfully expressed from pHES/HA1/STM1. The optimal expression condition was determined to be 37\(^\circ\)C in LB broth induced with 1.0 mM IPTG, and the maximum yield was reached at three hours post-induction.

The pHES plasmid encoded with the essential AT components derived from S. Typhimurium LT-2, hence, to determine the location of the fusion rHA1 expressed from pHES/HA1/STM1 clone, a cell membrane fractionation was performed. As described in Section 4.4.8. The pHES/HA1/STM1 clone was induced and harvested at three hours post-induction. The cells were sonicated and supernatant was subjected to several ultra-centrifugations using different buffers.

The procedure of membrane fractionation allowed the separation of total cell-lysate into soluble protein fraction, envelope protein fraction, non-integral outer membrane (OM) proteins and integral outer membrane proteins. The results showed that the pHES/rHA1 fusion protein was expressed as a part of STM1 integral outer membrane protein (Figure 4.26).
Figure 4.26: Membrane fractionation of induced pHES/HA1/STM1 and pHES/STM1 clones.

A) The immunoblot using 6x-His antibody, C) The immunoblot using PR-8 antiserum; Lane 1 to 4 and Lane 5 to 8 are samples from pHES/STM1 (negative control) and pHES/HA1/STM1, respectively. Lane M: Precision Plus Protein™ Kaleidoscope™ Standards, Lane 1 and 5: soluble protein fractions, Lane 2 to 4 and Lane 5 to 8, envelope protein fractions: inner membrane proteins, non-integral outer membrane proteins and integral outer membrane proteins respectively. B) and D) are the immunoblot of whole cell protein extracts from pHES/HA1/STM1 using 6x-His antibody and PR-8 antiserum, respectively. The results indicated that the rHA1 was expressed as a part of STM1 integral outer membrane protein.
4.4.9 pMOhly1/HA1/STM1 protein expression and characterisation

The detection of the pMOhly1/HA1 fusion protein from the pMOhly1 vector was difficult, as the level of expression from the haemolysin vector has been reported to be as little as 25 to 100 μg/L [390]. The STM1 harbouring the pMOhly1/HA1 and empty pMOhly1 were grown in BHI culture and the supernatant was tested for the expressed constructs.

Initially, trichloroacetic acid (TCA) precipitation was used to concentrate the fusion rHA1 from culture supernatants, but no protein of interest was detected from neither the SDS-PAGE nor immunoblot (data not shown).

Nickel-charged IMAC purification was then carried out (as described in Section 3.2.3). These samples were taken from both mid-log phase and overnight cultures. This method used the fused 6x-His tag to purify and concentrate the fusion protein in its native form. Fusion rHA1 was recovered using competitive elution with 250 mM of imidazole, and the samples were collected in 1 mL fractions. The samples were examined using SDS-PAGE coupled with silver staining and immunoblotting with PR-8 antiserum.

For the mid-log phase cultures, no fusion rHA1 was observed from the clone (data not shown). However, for the overnight cultures, a protein band at approximately 50 kDa was detected from the pMOhly1/HA1/STM1 clone, and the band was not seen in the pMOhly1/STM1 clone (Figure 4.27). In silico analysis predicted that the pMOhly1/HA1 fusion rHA1 has a MW of 49.04 kDa (prediction was performed using the ExPASy Compute pI/Mw tool) (Figure 4.28).
Figure 4.27: IMAC concentrated fractions of pMOhly1/HA1 fusion protein.

A) Silver stained SDS-PAGE gel, B) The immunoblot using PR-8 antiserum; Lane M: Precision Plus Protein™ All Blue Standards for A), Precision Plus Protein™ Kaleidoscope™ Standards for B), Lane 1 to 3: IMAC elution fractions (2nd to 4th mL) from pMOhly1/STM1 clone, Lane 4 to 6: IMAC elution fractions (2nd to 4th mL) from pMOhly1/HA1/STM1 clone. An unexpected protein band was also observed at approximately 45 kDa, which was purified by IMAC, but not reactive with PR-8 anti-serum. This suggested that this 45 kDa protein was not a breakdown product of the pMOhly1/HA1 fusion protein. It is possible that this protein is a stress-induced product, which has been expressed due to the expression of the pMOhly1/HA1 fusion protein. The yield of pMOhly1/HA1 was approximately 230 μg/L in overnight BHI broth.
Figure 4.28: The protein sequence of fusion rHA1 expressed in pMOhly1/HA1/STM1.

The fusion HA1 has an estimated MW of 49.04 kDa. **Red** coloured letters are the sequence encoding for influenza HA1 and the **purple** coloured letters are the 6x-His tag introduced to assist purification and detection.
4.5 Discussion

The MDCK cell-line was resuscitated from liquid nitrogen stocks and used to infect confluent MDCK cell-lines. The propagation of PR-8 virus in infected cell-lines was confirmed by the signs of cellular destruction and increased cell death using light inverted microscope.

The purification of PR-8 virus was straightforward and the purified virus samples were immediately stored at -80°C to prevent the degradation of influenza viral envelope, which is made up of host cell lipid-bilayer.

The extraction of influenza total RNA using TRIzol® LB reagent and subsequent cDNA synthesis were relatively simple and no obvious difficulties encountered during the procedure. However, great care was taken to prepare the workstation before commencing any procedure, and all procedures were conducted at low temperature to prevent cross contamination and degradation. Furthermore, the entire procedure from RNA extraction to PCR amplification using cDNA templates was performed within a day to minimise degradation.

Difficulties were encountered in the PCR amplification using insert primers. The initial attempt of setting the annealing temperature at 55°C did not result in any DNA products, and lowering the annealing temperature to 50°C resulted in an expected PCR product using pHE primer pair. The predicted Tm°C of thesis primers was above 60°C, therefore, it was surprising that a temperature lower than 55°C was required.

The insert primers for pMOhly1 were unable to generate any PCR product until the annealing temperature was lowered to 48°C. The excessive length of pMOhly1_SbfI_Rev primer may have contributed to the difficulty of generating PCR product using higher annealing
temperatures. The excessive length of the primer was required to introduce 6x-His tag into the pMOhly1 recombinant hlyA’ gene for protein detection and purification.

The sub-cloning of HA1 gene into pHES vector was achieved. The pHES/HA1 clone was confirmed using both enzymatic digestion to check for expected patterns, and also PCR to confirm. The plasmid construct was also amplified for sequence analysis, and the result indicated that the HA1 gene was inserted into pHES vector as expected. Two base-substitutions were observed in the inserted HA1 gene, and it was determined later that the mutations had always been in the virus stock and were not introduced during cloning procedure. One mutation was synonymous, and the other non-synonymous. The synonymous mutation resulted in an amino acid substitution from Proline to Serine in the PR-8 rHA1 protein sequence. However, the Serine substitution at the position does occur in other influenza strains such as (A/Cameron/JY2/1946(H1N1)).

In order to use the hly operon in its most efficient form, the NsiI site is the ideal place to insert a coding region to be expressed. PCR amplification proved useful in determining orientation of the insert. PCR determination of the orientation was achieved using different primer combinations by mixing pMOhly1 sequencing primers and the primers designed to elongate from the middle of the PR-8 HA1 gene. In theory, if the insert was present in the correct orientation there should be DNA products produced as listed with expected sizes. In the pMOhly1 screening, Clone No. 290 produced three bands and two of them were indicative of the correct orientation of the HA1 insert. In the screening of Clone No. 495, there was a product with a size of 1618 bp, indicating successful insertion of the gene. However, the absence of a PCR product when using primer combination SEQF_pMOhly1 + SEQR_PR8HA1, indicated incorrect orientation of the insert. Multiple bands were observed using primer combination SEQR_pMOhly1 + SEQF_PR8HA1 which can be attributed to
non-specific binding of the primers on the pMOhly1 plasmid. Though the PCR screening was at times unpredictable, it proved successful in identifying the correct pMOhly1/HA1 construct. Sequence analysis was also used to confirm the correct insertion of the influenza HA1 gene, and the same base-substitutions that appeared in the pHES/HA1 clone were also observed. The base-substitutions can be attributed to a single amino acid substitution, where the original Proline is substituted with Serine.

The passage of pHES/HA1 and pMOhly1/HA1 plasmid into vaccine strain STM1 was successful using PCR reactions. Using the primer combinations to confirm the correct insertion of the HA1 gene, the plasmid constructs isolated from bacterial vectors *E. coli* DH5-α, *S. Typhimurium* LT-2 and *S. Typhimurium* STM1 were shown to be the same for both pHES/HA1 and pMOhly1/HA1 plasmid constructs.

The purpose of using the pHES plasmid was to construct a vaccine carrier that displays the heterologous vaccine antigen on the bacterial surface. The pHES plasmid encodes a modified *ShdA* derived from *S. Typhimurium* LT-2, which is a known autotransporter gene.

The expression of recombinant *ShdA* is controlled by *lacIq*-Plac promoter. This promoter can be induced using IPTG and inhibited by glucose. Difficulties were encountered during pilot expression of the pHES/HA1 fusion protein, because even the presence of trace amount of glucose can still inhibit protein expression. However, an unexpected phenomenon was observed during the initial attempts of protein expression. It was found that if glucose was not added into the pre-expression overnight cultures, the expression became “leaky” (trace amount of expression), which resulted in inefficient induction of expression. Therefore, an extra wash step was included to remove glucose form the overnight pre-expression culture prior to induction.
Several parameters were considered in the optimisation of fusion pHES/HA1 expression, which included amount of inducing agent, expression temperature, expression media and a time-course study of the protein expression was performed to determine the optimal time to harvest clones for analysis. According to the results, the optimal expression condition was achieved in LB broth induced with 1.0 mM IPTG at 37°C. The amount of pHES rHA1 increased along the expression time-course, and reached its maximum at three hours post-induction. Among media tested for rHA1 expression, LB broth provided optimal conditions for expression followed by MH broth. BHI and NA failed to support the expression of pHES rHA1, which might be explained by the fact that these two media contain a substantial amount of glucose which would have inhibited rHA1 expression.

The location of pHES fusion rHA1 was determined using cell membrane fractionation. The procedure separates the total cell lysate into soluble and insoluble fractions, as the insoluble fraction mostly contained envelope proteins. The insoluble fraction was then treated with Triton X-100 and urea to dissolve some of the envelope proteins, and the remaining insoluble fraction was composed predominantly the outer membrane-bound proteins. As shown in Figure 4.26, a protein at the expected size of approximately 80 kDa was detected in the integral outer membrane fraction, which indicated that the pHES rHA1 was successfully expressed and translocated to the outer membrane of the vaccine strain STM1. The translocation of the recombinant passenger domain to the STM1 surface was as expected. This was similar to a previous report where it was demonstrated that the pHES plasmid encoding ShdA was able to express and translocate the passenger domain onto the E. coli surface [5].
Other than the use of the Salmonella ShdA gene for the purpose of surface displaying heterologous antigens, there are a number of other autotransporter genes which have been explored. Rizos et al., [391] examined the potential of using AIDA-I (E. coli adhesion involved in diffuse adherence) autotransporter domain to display antigenic fragments of the urease A subunit of H. pylori in an attenuated S. Typhimurium vaccine strain. Similar to the Salmonella ShdA autotransporter, AIDA-I consists of a 49 aa signal peptide, a 797 aa adhesion (passenger domain) and a 440 aa β-barrel domain anchored in the bacterial wall [392]. In this study, the fusion urease A subunit was successfully expressed and translocated on the Salmonella surface, and orally immunised mice exhibited reduced colonisation of H. pylori. In contrast to the strategy described in this chapter, Rizos and colleague incorporated a constitutive promoter for transcriptional control of the recombinant AIDA-I gene, and the resulting protein expression level appeared to be higher. However, the expression level of any recombinant protein is a multi-factor challenge in the development of such live vaccine carrier.

Charbit et al., 1987 [393] described a different strategy to display antigenic peptide at the surface of E. coli by using the outer membrane LamB protein as carrier. Although the experiment successfully elicited epitope-specific antibodies in an immunised animal model, there is a limitation in using such outer-membrane proteins as carrier. The use of outer-membrane proteins such as Omps, flagella and fimbriae for the purpose of surface display may be limited by the insert sizes, and the expression of globular-folded protein could inevitably disrupt normal functions of the Omp [394]. Autotransporters on the other hand, often have passenger domains over 100 kDa, an indication of that they are more feasible for expressing large inserts [395, 396].
The expression of fusion rHA1 was successful from the pMOhly1 plasmid in the vaccine strain STM1, but the level of expression was very low. The detection of rHA1 can only be achieved by concentrating the 6x-His tagged rHA1 using IMAC from 10 mL overnight culture. The concentrated sample could then be detected using SDS-PAGE stained with silver reagent and immunoblotting probed with PR-8 antiserum. Although pMOhly1 is a useful recombinant expression system, as it secretes protein from cells, it has been observed that expression levels can vary from 25 to 100 μg/L of culture supernatant [390].

Hahn and von Specht, 2003 [390] reported that culture size can significantly affected the protein expression rate in the haemolysin expression system due to the reduction in oxygen saturation levels in small culture volumes. This activation of the haemolysin system under reduced oxygen levels might be beneficial under in vivo conditions as the gastrointestinal tract is a low oxygen environment. It could result in a higher level of expression of the fusion products which could potentially increase the potency of the immune responses.

In general, extracellular expression of recombinant proteins has advantages in the development of live vaccine carriers. Currently, a large majority of recombinant proteins are expressed and secreted using the SecB-dependent type-II pathway. In this secretion pathway, prior secretion takes place; the pre-protein is first translocated across the inner membrane and folded in the periplasm, and then secreted into the culture medium through the means of non-specific periplasmic leakage [397]. This passive two-step secretion process can sometimes result in decreased extracellular yields due to partial secretion [398]. In contrast in the type I secretion system used in this study (E. coli α-haemolysin), the passenger protein is actively exported into the culture medium without accumulation in the periplasmic space [399, 400]. It is known that the expression level using α-haemolysin is low, however, some studies reported an increased translocation efficiency by introducing mutagenesis in HlyA,
HlyB and HlyD [401-403]. Sugamata and Toshikazu, 2005 [401] reported that some mutants created by random mutagenesis of HlyB exhibited increased secretion levels of a single-chain antibody-HlyA fusion protein for up to 27-fold compared to the wild-type α-haemolysin. Such techniques may be used in future studies to increase the protein expression and secretion level.

Currently, there are only a small number of studies that have successfully expressed recombinant proteins using the *E. coli* α-haemolysin in attenuated *Salmonella*. Orr *et al.*, 1999 [404] reported that low-level expression and secretion of a mutant Diphtheria toxin molecule (CRM197) was achieved in *S. Typhi* CVD908-htrA carrying pMOhly plasmid. Similar to what has been described in this Chapter, the gene encoding for the mutant Diphtheria toxin molecule was inserted into the *NsiI* site within the truncated *hlyA* gene. Another similar study conducted by Gentschev *et al.*, 1995 [405] also demonstrated the successful expression and secretion of Lysteriolysin in *S. dublin* aroAΔ by the same cloning strategy described in Orr *et al.*, 1999. Collectively, although extracellular secretion of recombinant protein could be achieved by using *E. coli* α-haemolysin, it is apparent that low-level expression of the recombinant protein is the major obstacle needed to be overcome for the broad application as a *Salmonella*-based live vaccine carrier.
4.6 Conclusion

The construction of both pHES/HA1 and pMOhly1/HA1 was successful, and the plasmid constructs were successfully transformed into the vaccine strain STM1.

The expression of pHES fusion rHA1 was achieved and the expression level was optimised for analysis and subsequent vaccine study. Furthermore, membrane fractionation confirmed the pHES fusion rHA1 was expressed and translocated to the outer membrane of the STM1. The expression of pMOhly1 fusion rHA1 was also successful, and the protein was detected in the supernatant of an overnight culture. Most importantly, both fusion proteins were detectable by PR-8 antiserum, indicating the presence of antigenic epitopes of the influenza HA. The immunogenicity of these clones was assessed in chickens, which is presented in Chapter 5.
Chapter 5

Vaccination of Chickens Using STM1 Expressing rHA1
5.1 Introduction

Avian influenza viruses are the cause of some of the most important diseases in the poultry industry which can also lead to spill-over infections into the human population. The rapid and continuous antigenic variation of the influenza virus has caused many episodes of widespread poultry epidemics in the past, and resulted in tremendous socioeconomic impact [172, 174, 406]. The currently utilised vaccines for the prevention of avian influenza in poultry are suboptimal [171, 257]. The conventional egg-based vaccines and *in vitro* expressed recombinant vaccine antigens require parenteral administration, and limited use has been reported due to high labour costs [260]. *In vivo* expression of influenza vaccine antigen using various viral and bacterial vectors has shown some success. However, pre-existing immunity to the vaccine carrier can significantly decrease the vaccine efficacy [162, 306].

*Salmonella* vaccine carriers may be used for large-scale immunisation in the poultry industry for the prevention of influenza infections via *in vivo* expression of influenza antigens. The intrinsic ability of *Salmonella* invasiveness allows it to provoke both B- and T-lymphocyte memory responses, hence inducing long-lasting mucosal, humoral and cell-mediated immunity [107]. After oral immunisation, *Salmonella* infiltrate through Microfold cells in the gut to gain entrance to the intestinal lining, and are taken-up by professional phagocytic cells, such as, macrophages and DCs, that disseminate to systemic compartments such as the liver and the spleen [379, 407].

After phagocytosis, *Salmonella* is found in vacuoles or phagosomes, and antigen presentation is predominantly restricted to the MHC class II pathway, eliciting primarily CD4+ Th1 and Th2 immune responses [408, 409] (Figure 5.1). In the case of viral infection, stimulated CD4+ Th1 cells are responsible for cytokine production including IFN-γ, IL-2 and TNF-α, which are
crucial for sustaining CD8\(^+\) T-lymphocyte responses; whilst CD4\(^+\) Th\(_2\) cells secrete IL-4 and IL-5 are important for the stimulation of humoral immune responses, all of which are essential in combating viral infections [354, 367, 410].

Several commercialised live-attenuated *Salmonella* vaccines exist, including STM1, which when used in chickens cause no signs of illness and can confer heterologous protection [320, 411-413]. Modification of these attenuated vaccines into vaccine carrier has been widely investigated using a varied assortment of heterologous pathogen antigens [3, 159, 160, 281, 309, 319, 414]. There have been various degrees of success using *Salmonella* as a vaccine carrier depending on the host animal and disease, including targeting avian influenza in chickens using hemagglutinin as a protective antigen [309, 310, 312].
In the endogenous pathway, antigens from intracellular pathogens, such as viruses, are degraded by the proteasome and the resulting peptides are loaded onto MHC class I molecules via the endoplasmic reticulum. The MHC class I complex is transported to the cell surface, where it stimulates CTLs. On the other hand, extracellular pathogens (such as *Salmonella*) are engulfed by phagosomes through the exogenous pathway. After phagosomal processing, the pathogen-derived peptides are loaded onto MHC class II, and after being transported for presentation, it stimulates CD4\(^+\) Th\(_1\) and Th\(_2\) immune responses, leading to the production of cytokines and antibodies, which are essential for sustaining CD8\(^+\) T-lymphocyte and humoral immune responses. Furthermore, other than the classical pathways described above, dendritic cells can present exogenously processed antigens on MHC class I molecules via a process known as “cross-presentation”. Image modified from [415].
Several factors may influence the efficacy and type of immune response induced against heterologous passenger antigen. For instance, the translocation of heterologous antigen after expression may vary the immunogenicity of such an *in vivo* expressed antigen for induction of humoral immunity. It is important that the heterologous antigen can translocate into the periplasmic membrane or outer membrane surface, or even be secreted into the extracellular space. Another factor that can play a vital role is the type of mutation engineered to attenuate the vector vaccine. Some mutations that lead to the disruption of genes encoding for essential metabolic enzymes for the survival of a microbe *in vivo* result in the generation of mutants which are safe and have minimal survival in the host. However, such mutations may have an impact on the course of infection, hence changing the quality of the induced immune response [129]. Furthermore, the expression of heterologous antigen may result in unnecessary metabolic burden to the vaccine vector, and lead to over-attenuation of the organism [129].

One potential impediment for the use of *Salmonella* vaccine carrier is pre-existing immunity as a result of pre-exposure to either a heterologous or homologous strain. For chickens, *Salmonella* exposure can occur via both horizontal and vertical transmission and many different strategies have been used to reduce transmission including feed and drinking water acidification, immune strategies based on passive and active immunity, nutrient composition to reduced susceptibility to *Salmonella* and improve hygiene practices [416].

Currently, there is speculation over the effect of pre-existing immunity on heterologous delivered antigens. There have been various reports that conclude that pre-existing immunity leads to up-regulation of the immune response to the delivered antigen [2, 4, 417-422]. However, there have been some contradictory reports that pre-existing immunity reduces heterologous antigen-specific immune responses and that this effect is a restrictive
limitation of this delivery system as exposure to *Salmonella* is common [423-426].

In the previous chapter, work leading to the generation of a set of recombinant STM1 vaccine strains was described. The aim of the work described in this chapter is to evaluate the ability of the *Salmonella* STM1 vaccine to deliver heterologous antigen *in vivo* and elicit both cellular and humoral immune responses. Influenza hemagglutinin domain 1 was used as the heterologous antigen, in which, the pHES/HA1/STM1 strains were engineered to translocate and display the rHA1 on STM1 outer membrane, and pMOhly1/HA1/STM1 strains were equipped with the ability to secrete rHA1 into extracellular space.

The aim of work described in this chapter was as follows:

- To evaluate the plasmid stability of STM1 harbouring influenza HA1 *in vivo* after being orally administered in chickens.
- To evaluate the immune responses of the STM1 vaccine strains harbouring influenza HA1 in a chicken trial.
5.2 Materials and methods

5.2.1 Chickens and bacterial strains

Newly hatched mix-gender Broiler chicks were obtained from Inghams Farms, Pakenham, Victoria. The recombinant STM1 clones used in the immunisation study were described in Chapter 4. These included pHES/HA1/STM1 and pMOhly1/HA1/STM1 carrying influenza HA1 gene in the pHES and pMOhly1 vector, respectively, and the negative controls pHES/STM1 and pMOhly1/STM1 carrying the expression vector only.

5.2.2 Vaccine preparation

Prior to preparation, all clones were confirmed using PCR amplification with primer combinations as listed in Table 4.1 and Table 4.2.

For pHES/HA1/STM1 and pHES/STM1 vaccines, the clones were expressed in LB broth induced with 1.0 mM IPTG at 37°C for three hours with 180 rpm orbital shaking. The cells were washed three times with sterile ice-cold PBS, and diluted to 1 x 10^9 CFU/mL for administration. For pMOhly1/HA1/STM1 and pMOhly1/STM1 vaccines, the clones were cultured in BHI broth at 37°C for 16 to 18 hours with 180 rpm orbital shaking. Cells were washed as described above for pHES clones.

5.2.3 Immunisation of chickens via oral route

The immunogenicity of the vaccine strains pHES/HA1/STM1 and pMOhly1/HA1/STM1, and negative control strains pHES/STM1 and pMOhly1/STM1 was determined in a chicken. A total of 20 newly-hatched mix-gender chicks were divided into four groups (Table 5.1), where each chicken was vaccinated twice at 7 and 22 days of age with STM1 expressing influenza HA1 either from pHES or pMOhly1 plasmid, and STM1 vector carrying empty pHES
or pMOhly1 plasmids as a negative control. Vaccines were given via oral gavaging at $1 \times 10^9$ CFU vaccine STM1 per chicken. Chicken faecal samples were collected one day prior the first immunisation and every other day thereafter throughout the study for assessing vector retention and IgA extraction. Chickens were decapitated two weeks after the second immunisation, and serum samples and spleens were collected for immunological assays. Prior to decapitation, the chickens had undergone anaesthesia via an intramuscular injection into the pectoral muscle using a cocktail mix of 1.0 mg Ketamine (Parnell, Australia) and 2.0 mg Xylazine (Troy Laboratories, Australia) per 1 Kg chicken.

### 5.2.4 Detection of STM1 vaccine strains from faecal samples

Eight fresh faecal samples were collected from each experimental group one day prior to the first vaccination and every second day thereafter. Each faecal sample was collected and stored at $4^\circ$C until processed. For the pre-vaccination samples, the samples were streaked onto selective media XLD agar (Oxoid, UK), to determine whether the chicks had been exposed to *Salmonella*. Vaccine constructs were isolated on Ampicillin (100 μg/mL) supplemented LB media for pMOhly1 clones, and Chloramphenicol (40 μg/mL) and glucose (2% w/v) supplemented LB media for pHES clones. The isolated clones were confirmed by colony PCR.
Table 5.1: Vaccine trial configuration.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Vaccination</th>
<th>Number of chicks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>pHES/STM1: STM1 harbouring empty pHES plasmid</td>
<td>5</td>
</tr>
<tr>
<td>Group 2</td>
<td>pHES/HA1/STM1: STM1 expressing HA1 using pHES plasmid vector</td>
<td>5</td>
</tr>
<tr>
<td>Group 3</td>
<td>pMOhly1/STM1: STM1 harbouring empty pMOhly1 plasmid</td>
<td>4**</td>
</tr>
<tr>
<td>Group 4</td>
<td>pMOhly1/HA1/STM1: STM1 expressing HA1 using pMOhly1 plasmid vector</td>
<td>5</td>
</tr>
</tbody>
</table>

Group 1 and 3 were negative controls, group 1 chickens were vaccinated with STM1 harbouring empty pHES plasmid and group 3 chickens were vaccinated with STM1 harbouring empty pMOhly1 plasmid. The evaluation of humoral and cell-mediated immune responses from group 2 and 4 were compared with these negative controls accordingly.

** One chick was found unhealthy and subsequently euthanised on welfare grounds.
5.2.5 Preparation of faecal samples for secretory IgA analysis

Faecal samples were collected within minutes of defecation over a period of an hour. Samples were weighed and processed immediately. Standard faecal preparations of 1:10 dilution (w/v) were made in sterile PBS, and the samples were vortexed vigorously for 5 minutes and allowed to stand at room temperature for one hour [427]. After centrifugation at 15,000 x g for 15 minutes, the supernatant was collected and stored at -20°C until analysis.

Prior to analysis, the samples were thawed at room temperature and added to the ELISA plate in triplicate. Wells were coated with either 10 μg/mL of purified rHA1 expressed from Pichia or 5 μg/mL heat-inactivated influenza PR-8 virus [428]. Two- or four-fold dilution of faecal extract obtained from vaccinated chickens were used as primary antibody, and goat anti-chicken IgA-HRP (Abcam®, USA) diluted 1:5000 in diluent (PBST/1% (w/v) skim milk), was used as secondary conjugated antibody.

5.2.6 ELISA protocols for chicken IgG

Serum antibody responses of vaccinated chickens were measured using the ELISA as outlined in Section 2.7.5 with a few modifications. Wells were coated with either Pichia-expressed rHA1 or heat-inactivated influenza PR-8 virus as described above. Two- or four-fold dilutions of sera obtained from vaccinated chickens were used as primary antibody, and Goat anti-rabbit IgG (whole molecule)-HRP (Sigma-Aldrich, USA) diluted 1:5000 in diluent, was used as secondary conjugated antibody. Following the addition of the substrate TMB, plates were incubated for 15 to 30 minutes before the addition of 50 μL of a 1 M solution of sulphuric acid. The endpoint was determined as the dilution at which the optical density (OD) at a wavelength of 450 nm (OD₄₅₀) was three times the background level.
5.2.7 Hemagglutination Inhibition assay

HI titre of vaccinated chickens was measured using the protocol outlined in Section 3.2.9.3.2, and both serum IgG and secretory IgA samples were examined for specific virus-neutralising antibody responses from immunised chickens.

5.2.8 ELISpot assay

5.2.8.1 Media, buffers and solutions

5.2.8.1.1 Media

**Blocking medium:** RPMI1640 (Gibco®, USA) supplemented with 2% (v/v) Fetal Calf Serum (FCS) (Gibco®, USA) and 100 U/mL Pen/Strep (Gibco®, USA).

**Collection medium:** Same as blocking medium

**Culture medium:** RPMI1640 supplemented with 10% (v/v) FCS, 100 U/mL Pen/Strep, and 50 μM β-mercaptoethanol (Gibco®, USA).

5.2.8.1.2 Buffers and solutions

**Assay buffer:** PBST added with 1% (w/v) BSA.

**Carbonate buffer 1:** 1 M NaHCO$_3$ in Milli Q water. Filter sterilised and stored at 4°C.

**Carbonate buffer 2:** 1 M Na$_2$CO$_3$ in Milli Q water. Filter sterilised and stored at 4°C.

**ELISpot coating buffer:** combine 3.4 mL Carbonate buffer 1 and 1.5 mL Carbonate buffer 2 in 100 mL sterile Milli Q water. Adjust to pH9.6 using either Carbonate buffer 1, or Carbonate buffer 2. Made for immediate use.

**1X Dulbecco’s Phosphate Buffer Saline:** 10-fold dilution of 10X DPBS (Gibco®, USA) in sterile Milli Q water.
5.2.8.1.3 IFN-γ chicken antibody pair

Chicken IFN-γ levels were determined using the IFN-γ Chicken Antibody Pair Kit (CAC1233, Life Technologies, USA). This kit includes Anti-Chicken IFN-γ, Anti-Chicken IFN-γ Biotin and Streptavidin-HRP.

5.2.8.2 Preparation of ELISpot plates

Multiscreen™-IP 96-well ELISpot plates (Merck, USA) were prepared by wetting the membranes with sterile 35% ethanol, followed by three washes with sterile 1X DPBS. Plates were coated for 24 hours at 4°C with 100 μL anti-chicken IFN-γ antibody (5.0 μL/mL) in ELISpot coating buffer; the wells were washed twice with Blocking medium and patted dry with autoclaved paper towelling. Plates were blocked with 200 μL of Blocking medium for one hour at 37°C supplemented with 5% CO₂. A volume of 100 μL of Culture medium was added to each well whilst the splenocyte samples were prepared.

5.2.8.3 Collection and preparation of chicken splenocytes

Upon necropsy, chicken spleens were removed aseptically and placed in Collection buffer and stored on ice until process. The chicken splenocytes were homogenised by squashing the spleen using the back of a 3 mL syringe. The homogenised cells were resuspended in 5 mL of Collection medium and filtered through a cell strainer (70 μm) (BD Biosciences, USA). Splenocytes were isolated by density-gradient centrifugation at 850 x g for 20 minutes in Histopaque®-1077 (Sigma-Aldrich, USA), and cells were washed once in 1X DPBS and twice in Culture medium, and resuspended in Culture medium. Cell viability was calculated using Trypan Blue exclusion. Ten microlitres of cell suspension, 10 μL of Trypan Blue (Sigma-Aldrich, Australia) and 80 μL of 1X DPBS were mixed and viable cells counted in a Countess Automated Cell Counter (Life Technologies, USA). Cell concentration was adjusted to 1 x 10⁶ cells in 180 μL Culture medium. The splenocyte suspension was used to determine chicken
5.2.8.4 Determination of chicken IFN-γ levels

Upon seeding prepared splenocytes, the Culture medium was discarded, plates were patted dry and 180 μL of splenocytes assed to each well (containing $1 \times 10^6$ cells/180 μL). All standard and sample wells were seeded in triplicate, as well as three wells per control. The addition of 20 μL of a stock (10 μg/mL) of Concanavalin A (Sigma-Aldrich, Germany), a mitogen that would serve as a positive control, 50 μg per well BSA would serve as a negative unrelated protein control and 20 μL of Culture medium to confirm that there was no contamination of the tissue culture media. Two sets of stimuli were prepared in Culture medium, which includes the purified Pichia-expressed rHA1 protein at a concentration of 250 μg/mL and 20 μL added to each sample well, and heat-inactivated influenza PR-8 virus [428] at a concentration of 50 μg/mL and 20 μL added to each sample well. The plates were incubated in a humidified incubator at 41°C supplemented with 5% CO₂ for 48 hours.

Subsequently, the plates were washed six times with PBST and once with PBS, 50 μL of Detection Biotinylated anti-chicken IFN-γ in Assay buffer at a concentration of 0.5 μg/mL was added to each well and the plate incubated at room temperature for two hours with 100 rpm shaking. The plates were washed five times with PBST and once with PBS. To each well 100 μL of 2 μg/mL Streptavidin-alkaline phosphatase (Life Technologies, USA) was added and the plates were incubated at room temperature for one hour in dark at room temperature. The plates were washed five times with PBST and once with PBS and patted dry with autoclaved paper towelling. To develop the assay, 50 μL of BCIP®/NBT (Sigma-Aldrich, USA) was added to each well and incubated at room temperature with gentle continual shaking for 30 minutes in dark. The assay was stopped by washing the plates with plenty of water and allowed the plates to dry before analysis. The spots were counted under a dissecting microscope.
5.2.9 Statistical analysis

All statistical analysis was calculated using the Microsoft Excel 2007 using the Data Analysis add-in. The results displaying a normal distribution and equal variances were analysed using Student’s T-test.
5.3 Results

5.3.1 Immunisation of chickens

Prior to the first immunisation, one chick was found unhealthy and subsequently euthanised on welfare grounds by the RMIT Animal Ethics Committee – Animal Welfare Officer. A necropsy was carried out and the result indicated inappetence. Professional advice suggested that it is not unusual in young chick flocks, as supplementary food is already provided and no further investigation was taken. A group of four chicks was used for immunisation with STM1 carrying pMOhly1 empty plasmid.

5.3.2 Recovery of STM1 vaccine plasmids from faecal samples

Pre-immunisation samples were streaked onto XLD agar to check for pre-exposure/colonisation of Salmonella serovar strains [429, 430]. No typical S. Typhimurium strains were found in the faecal samples collected prior to immunisation.

In order to determine how successfully the constructs are retained after immunisation, the percentages of STM1 vaccine constructs were determined from freshly defecated faecal samples. The result is presented as percentages of positive detection of vaccine constructs using antibiotic-containing LB agar and PCR confirmation along the course of the trial.

For pHES/STM1 and pHES/HA1/STM1 vaccines, results indicated that neither pHES vaccine constructs can retain plasmid stability immediately after immunisation (data not shown) as neither the antibiotic-containing agar plates nor the PCR reactions detected pHES plasmids. However, pMOhly1/STM1 and pMOhly1/HA1/STM1 vaccine plasmids were detectible for up to seven days after the first immunisation and up to six days after the second immunisation in collected faecal samples (Figure 5.2). An overall decreased positive detection rate of
pMOhly1 vaccine constructs after the second immunisation was also observed. The highest
detection rate of pMOhly1/STM1 and pMOhly1/HA1/STM1 after the first immunisation was
88% (7/8) and 75% (6/8) respectively, and after the second immunisation was 75% (6/8) and
38% (3/8) respectively.
Figure 5.2: Detection of pMOhly1/STM1 and pMOhly1/HA1/STM1 vaccine constructs from faecal samples along the course of the trial.

According to the results collected, the pMOhly1 vaccine constructs were able to persist *in vivo* for up to seven days after immunisation, and the overall positive detection rate of the vaccine constructs were higher after the first immunisation than the second immunisation.
5.3.3 Humoral immune responses

In order to determine the immunogenicity of the STM1 vaccine strains, HA1-specific and virus-specific humoral responses were measured using ELISA.

Sera collected upon decapitation was analysed for the presence of IgG humoral responses specific to both the Pichia-expressed rHA1 and inactivated virion of influenza PR-8. Neither pHES/HA1/STM1 (Figure 5.3A & Figure 5.3B) nor pMOhly1/HA1/STM1 (Figure 5.4A & Figure 5.4B) vaccinated groups showed elevated immune responses against rHA1 or inactivated PR-8 virion over the control groups.

Similarly faecal IgA collected prior to decapitation did not show any specific immune response against pHES/HA1/STM1 (Figure 5.5A & Figure 5.5B) or pMOhly1/HA1/STM1 (Figure 5.6A & Figure 5.6B). Furthermore no statistically significant differences were found between samples collected prior to the first immunisation and prior to decapitation (data not shown).

Specific virus-neutralising humoral responses of the collected serum IgG and faecal IgA were determined using HI assay. However, no inhibition of hemagglutination between PR-8 virus and human red blood cells was observed (data not shown).

In conclusion, neither the pHES/HA1/STM1 and pMOhly1/HA1/STM1 vaccine strains were able to elicit specific humoral responses against Pichia-expressed rHA1 or the influenza PR-8 virion.
Figure 5.3: IgG response of pHES/HA1/STM1 vaccinated groups.

A) specific IgG response against PR-8 virion; B) specific IgG response against *Pichia*-expressed rHA1. The results showed no significant difference between the pHES/HA1/STM1 vaccinated group over the control group at any given dilution factor ($P > 0.1$).
Figure 5.4: IgG response of pMOhly1/HA1/STM1 vaccinated groups.

A) specific IgG response against PR-8 virion; B) specific IgG response against *Pichia*-expressed rHA1. The results showed no significant difference between the pMOhly1/HA1/STM1 vaccinated group over the control group at any given dilution factor (*P* > 0.1).
Figure 5.5: IgA response of pHES/HA1/STM1 vaccinated groups.

A) specific IgA response against PR-8 virion; B) specific IgA response against *Pichia*-expressed rHA1. The results showed no statistically significant differences between the pHES/HA1/STM1 vaccinated group over the pHES/STM1 control group at any given dilution factor ($P>0.1$).
Figure 5.6: IgA response of pMOhly1/HA1/STM1 vaccinated groups.

A) specific IgA response against PR-8 virion; B) specific IgA response against *Pichia*-expressed rHA1. The results showed no statistically significant differences between the pMOhly1/HA1/STM1 vaccinated group over the control group at any given dilution factor ($P>0.1$).
5.3.4 Cell-mediated immune responses

Chickens were sacrificed on the 35th day, which was two weeks after the second dose of vaccination. Splenocytes were prepared for the ELISpot assay as described in Section 5.2.8.3. The number of cytokine secreting cells in each group was compared using Student’s T tests. Results were reported as spot count with standard deviation (spot count + S.D.). Cell-mediated immune responses were measured by the production of IFN-γ by splenocytes in response to stimulation by either inactivated PR-8 virion and Pichia-expressed rHA1, and BSA as an internal negative control. Concanavalin A, a plant-based mitogen known to stimulate T-lymphocyte proliferation served as an internal positive control.

Comparative analysis between chickens vaccinated with pHES/STM1 and pHES/HA1/STM1 indicated that STM1 expressing influenza HA1 using pHES-vectored construct did not increase IFN-γ secretion following stimulation with either inactivated PR-8 virion (P>0.10) or Pichia-expressed rHA1 (P>0.10) (Figure 5.7).

Analysis of IFN-γ secreting splenocytes obtained from chickens vaccinated with pMOhly1/STM1 and pMOhly1/HA1/STM1 indicated a statistically significant response in splenocytes stimulated with inactivated PR-8 virion (P<0.01) and in splenocytes stimulated with Pichia-expressed rHA1 (P<0.05) when compared with the control as shown in Figure 5.8.

These results indicate that STM1 expressing influenza HA1 using the pHES-vectored construct cannot induce an IFN-γ response, but pMOhly1-vectored construct can induce an IFN-γ response. In addition, splenocytes stimulated with inactivated PR-8 virion exhibited slightly elevated IFN-γ secretion compared to rHA1 stimulated splenocytes. Increased IFN-γ secretion might be induced by other immunostimulating components carried by the virion.
Figure 5.7: ELISpot assay of IFN-γ secreting T-lymphocytes from pHES/HA1/STM1 vaccinated chickens.

Splenocytes from vaccinated chickens were stimulated with *Pichia*-expressed rHA1 or inactivated PR-8 virion, the number of positive IFN-γ secreting cells determined. Compared with the control group, chickens vaccinated with pHES/HA1/STM1 was unable to induce any statistically significantly different IFN-γ responses. (*P>0.10)
Figure 5.8: ELISpot assay of IFN-γ secreting T-lymphocytes from pMOhly1/HA1/STM1 vaccinated chickens.

Splenocytes from vaccinated chickens were stimulated with *Pichia*-expressed rHA1 or inactivated PR-8 virion, the number of positive IFN-γ secreting cells determined. Compared with the control group, chickens vaccinated with pMOhly1/HA1/STM1 construct yielded significantly higher spot counts.

(**$P<0.05$, ***$P<0.01$)
5.4 Discussion

The use of live attenuated Salmonella as a vector to deliver heterologous antigen has been previously demonstrated [3, 150, 431]. The ability of the Salmonella vectors to target both cell-mediated and humoral immune responses and present heterologous antigen makes them an ideal candidate for the delivery of passenger antigen and induction of long-lasting immune responses [151, 152, 158]. The use of Salmonella-vectored vaccine carrier to deliver heterologous antigens for the prevention of avian influenza virus has been studied to a lesser extent, and currently there are no conclusive results available for potential commercialisation [311, 312, 314].

The aim of the work described in this chapter was to analyse the optimal antigen presentation of influenza HA1 in vivo from Salmonella STM1 in order to characterise the optimal expression system which can be further utilised for the efficient delivery of influenza HA1 by using STM1 as a vector.

As described in the previous chapter, pHES and pMOhly1 vectors were engineered to express influenza HA1 in Salmonella STM1 and present these passenger proteins to different compartments for optimal immunogenicity. pHES encodes a modified ShdA autotransporter gene derived from S. Typhimurium LT-2, and the HA1 gene was cloned immediately downstream of N-terminal signal peptide for surface display of the fusion rHA1 protein after expression. pMOhly1 encodes the necessary components of the E. coli α-haemolysin secretion system, which secrete the fusion rHA1 into the extracellular compartment.

Animal experiments demonstrated that STM1 expressing HA1 using the pHES vector loses its plasmid stability immediately after oral administration; whereas STM1 carrying the pMOhly1 construct remained detectable for up to seven days after administration. For pHES-vectored
STM1 vaccines, the increased metabolic burden due to carriage of the pHES plasmid may have played an important role to the spontaneous loss of plasmid [129], which is not uncommon when the metabolic burden associated with plasmid replication over-attenuates the vaccine carrier [130]. Another possible explanation to the lack of plasmid detection after administration is the alteration of the surface-protein composition due to carriage of pHES vector, which may have had a significant impact on the retention of the vaccine carrier. It has been demonstrated in a mouse model that a serotype of S. Typhimurium strains harbouring mutated ShdA has reduced ability for intestinal persistence with reduced number and shorter period of faeces shedding compared to its isogenic parent strain [383, 386, 432]. Therefore, it is possible that the STM1 displaying a modified ShdA autotransporter protein on the surface hindered the vaccine’s ability to persist in vivo. Furthermore, as an IPTG-inducible plasmid vector, the fusion rHA1 could only be presented to chicken immune systems in a one-off fashion, which was achieved by IPTG-induction prior to administration. Take together, the spontaneous loss of plasmid, inability to persist inside chicken intestinal tract, and lack of repeated antigen presentation to chicken immune systems, the STM1 expressing HA1 using pHES vector was unable to elicit any HA1-specific immune responses.

The STM1 expressing fusion rHA1 using the pMOhly1 vector, which was examined in this animal trial, has been shown to not induce any humoral immune responses including HA1-specific serum IgG, secretory IgA nor virus neutralising serum antibody response. However, cell-mediated immune response, as measured by enumeration of IFN-γ secreting splenocytes indicated significant elevation when stimulated by either Pichia-expressed rHA1 (P<0.05) or inactivated PR-8 virion (P<0.01) compared with the control group.

These findings indicate that the STM1 expressing HA1 using the pMOhly1 vector was unable to elicit antibody-mediated immune responses against influenza HA1. In contrast to our
findings, Liljebjelk and colleagues used a plasmid-based expression system, expressing
codon-optimised H5N1 HA gene under the control of an anaerobically induced promoter in a
S. Typhimurium Δaro mutant [312]. They found moderately elevated protective humoral
immune responses in chickens via mucosal immunisation suggesting that the use of an in vivo inducible promoter, such as nir15, can potentially overcome the problem of toxicity of
over expressed foreign protein or loss of expression plasmid. However, pMOhly1-vectored
STM1 vaccines secrete foreign protein extracellularly, hence the accumulation of
heterologous toxicity in the vaccine carrier is unlikely. One foreseeable disadvantage of using
constitutive vectors for the purpose of delivering vaccine antigen is the continuous metabolic
burden associated with protein expression, which could potentially over-attenuate the
vaccine carrier, hence retarding vaccine immunogenicity. Furthermore, it has been reported
by Hahn and von Specht, 2003 [390] that the level of protein expression from the haemolysin
vector can be as low as 25 to 100 μg/L.

Unlike the animal trial described in Chapter 3, where robust HA1-specific immune responses
were elicited by subcutaneous administration of adjuvanted 25 μg of purified influenza HA1
protein. The STM1 vaccine carriers expressing HA1 were unable to elicit any humoral
immune responses.

Evaluation of cell-mediated immune response from stimulated splenocytes indicated a
positive response for the secretion of IFN-γ in the chickens vaccinated with STM1 expressing
HA1 from pMOhly1 plasmid when compared with the control group, indicating that the rHA1
antigen may be presented in a way to preferentially induce T-lymphocytes that secrete IFN-γ,
indicative of a Th1-restricted type response in chickens.
As *S. Typhimurium* is a facultative intracellular pathogen, T-lymphocytes are essential components of the specific immune response. The bacterium-specific CD4\(^+\) and CD8\(^+\) T-lymphocytes are critical for a complete immune response against *S. Typhimurium* [121]. Following entry into the gut, the vaccine organisms are taken up by phagocytic cells, and the fusion rHA1 will be processed through the exogenous pathways. The exogenous pathway predominately presents antigen through MHC class II, leading to the activation of CD4\(^+\) T helper lymphocyte responses [415]. As shown in the results, the STM1 expressing HA1 from pMOhly1 plasmid induced a Th\(_1\) response, which is essential for the control of influenza infection by sustaining CD8\(^+\) T-lymphocyte response. It was unfortunate that Th\(_2\)-type immune responses were not evaluated in this study due to unavailability of an anti-chicken IL-4 antibody for the ELISpot assay. However, the lack of antigen-specific humoral immune response suggests the lack of Th\(_2\) elicitation. Several factors such as type of *Salmonella* strains, type of mutation and the type of heterologous protein expressed play a decisive role in the generation of complete cell-mediated immune responses [433, 434].

Another possible explanation to the sole production of low level IFN-\(\gamma\) is that, the STM1 expressed pMOhly1/HA1 was processed through the endogenous pathway and directly presented by MHC class I molecules or cross-presented from the exogenous pathway, resulting in the activation of CD8\(^+\) Tc\(_1\) cells. Cross-presentation of exogenous antigens on MHC class I molecules is known to play an important role in the initiation of CD8\(^+\) T-lymphocyte responses [435]. The process is mainly carried out by specific dendritic cell subsets through an adaption of their endocytic and phagocytic pathways. Chin’ombe *et al.*, 2009 [436] reported a successful induction of CD8\(^+\) T-lymphocyte cytokine responses in orally immunised mice using attenuated an *Salmonella* vaccine strain expressing a green fluorescent protein model antigen. Such results highlight the potential of using attenuated *Salmonella* vaccine to induce antigen-specific CD8\(^+\) responses. The mechanisms of
Salmonella-expressed antigen being presented to the immune system by the MHC class I pathway to induce CD8\(^+\) responses is not yet fully understood. Nevertheless, it has been noticed that Salmonella have high tropism for DCs and these DCs have the ability to cross-prime exogenous antigens for induction of CD8\(^+\) T-lymphocyte responses [437-442]. Furthermore, DCs can also engulf the apoptotic cells from Salmonella infection, which may serve as another source of antigen that can be processed for the induction of CD8\(^+\) T-lymphocyte responses [441, 442]. Moreover, it has been reported that, during Salmonella infection, the Salmonella LPS stimulates TLRs on DCs, which leads to the production of IL-12 [443]. IL-12 promotes IFN-\(\gamma\) production and activation of CD4\(^+\) Th\(_1\) responses. Therefore, it is possible that the presence of IL-12 induced by Salmonella LPS enables the STM1-expressed HA1 to stimulate Th\(_1\)-type immune responses.

It was observed that splenocytes stimulated with inactivated PR-8 virion exhibited slightly elevated IFN-\(\gamma\) secretion compared to rHA1 stimulated splenocytes. Butchko et al., 1978 [444] reported that non-infectious influenza virus type A strain H2N2 is a strong T- and B-lymphocyte mitogen. Furthermore, Anders et al., 1984 [445] showed that inactivated influenza type A subtype H1 induced low, but significant levels of T-lymphocyte proliferation in unprimed splenocytes from BALB/c mice. Further investigation by the authors demonstrated the virus-induced mitogenesis can be inhibited by monoclonal antibodies directed against hemagglutinin, which strongly suggests the mitogenic property of influenza hemagglutinin [445].

This study has shown that although attenuated S. Typhimurium STM1 holds many promising factors to be used as vaccine carrier, including elicitation of both CD4\(^+\) and CD8\(^+\) T-lymphocyte responses, the immunogenicity of such a system heavily depends on how the heterologous protein is expressed and delivered to the host. STM1 expressing fusion rHA1
using pHES vector has been shown to be ineffective in stimulating humoral and cell-mediated immune responses. It is possible that the inability to persist \textit{in vivo} combined with the lack of repeated antigen presentation may contribute to this finding. However, STM1 expressing HA1 using pMOhy1 vector was able to elicit Th$_1$-biased immune responses, but no humoral immune responses.

Based on the findings of this chapter it remains possible that STM1 mutants can be used effectively as a carrier to deliver influenza antigen and confer protective immunity through oral immunisation. Nevertheless, the efficacy of such vaccine still requires enhancements by in-depth understanding of the host-to-vaccine interactions and mechanisms to increase the level of protein expression.
Chapter 6

Conclusion
Avian influenza virus is a continuous and re-emerging disease with a devastating impact on the global poultry industry and occasionally to the human population [163, 164]. Currently, the poultry industry heavily relies on enhancing biosecurity measures, frequent surveillance and restricting poultry movement to control and prevent an influenza epidemic [170, 177]. However, these basic control measures have failed to effectively control the virus spread, as sporadic outbreaks of avian influenza infections still happen on yearly basis [257]. In recent years, the introduction of prophylaxis vaccines for the prevention of influenza infections in the poultry industry have helped to limit the socioeconomic impact during these outbreaks, but several limitations and concerns associated with vaccine efficiency has been debated in the scientific community [169, 282]. Several noticeable limitations have resulted in suboptimal coverage of these vaccines, including the time-consuming production of the egg-based conventional inactivated whole-virus vaccine and labour-intensive parenteral administration [253]. The development of an efficacious vaccine to protect against avian influenza virus would improve the husbandry of poultry and increase the productivity of poultry farm, and most importantly minimise spill-over infections into the human population [170, 213].

This study focused on using two different vaccine approaches to elicit immune responses that would be protective against influenza infections; the use of yeast-expressed influenza rHA1 protein as a subunit vaccine antigen, and the use of rHA1 protein delivered by attenuated Salmonella vectors. The advantage of using yeast-expressed influenza rHA1 as a subunit vaccine is its scalability and productivity [290]; whereas Salmonella-vectored influenza vaccine can be administered simply by mixing with drinking water or aerosol spray [98].
The codon-optimised influenza PR-8 HA1 gene was engineered for expression from *P. pastoris* as a soluble secretory molecule. The expression studies indicated that rHA1 was successfully expressed and secreted into the growth medium as soluble molecule, and the rHA1 yield was the highest concentration in BMMY medium after 72 hours growth. This result suggested the buffering mechanism and additional nutrients supplied in yeast extract and peptone were crucial for optimal expression of rHA1. In optimised expression conditions, the total rHA1 protein yield was approximately 2.9 mg/L.

The animal experiment conducted using rHA1 as subunit vaccine antigen indicated the successful elicitation of both humoral and cell-mediated immune responses. Immunised mice exhibited significantly elevated IgG responses specific to purified rHA1, and endpoint titres of 51200 were obtained from ELISA when compared with the control group. The virus-neutralising HI titre of the immunised mice was determined to be $7.4\log_2 \pm 5.5\log_2$; whereas serum collected from the control group exhibited no virus-neutralising ability.

In an investigation conducted by Saelens *et al.*, 1999 [235], where the entire influenza A/Victoria/3/75 (H3N2 subtype) HA gene was expressed from *P. pastoris*, immunised mice exhibited an elicitation of virus-specific IgG responses and complete survival from homologous 10 LD$_{50}$ challenge. Our investigation has shown that the globular HA1 domain of the entire HA protein is enough to stimulate the production of a virus-specific IgG immune response, and also the induction of virus-neutralising antibody responses.

Cell-mediated immune responses were examined using the ELISpot assay, and the results suggested a T-lymphocyte bias towards Th$_2$ immunity, indicated by the elevated secretion of IL-4 from stimulated splenocytes. Splenocytes collected from the immunised mice failed to demonstrate statistically significant IFN-γ secretion when compared with the control group,
indicating the lack of Th1 immune elicitation. Such results suggested the rHA1 subunit vaccine would induce an antibody-mediated immune response. In the case of influenza virus infection, robust Th2 responses are crucial for the prevention of viral attachment and entry by the production of neutralising antibodies [446]. The lack of Th1 elicitation might result in the absence of CD8$^+$ T-lymphocyte activation. Nevertheless, literature has suggested that CD8$^+$ T-lymphocyte responses are predominantly responsible for delayed-type viral clearance rather than the prevention of viral infection [7, 297, 447]. Therefore, the inability to generate protective cytotoxic T-lymphocyte responses in mice does not necessarily mean the vaccine will not be protective against influenza infection.

Live attenuated *Salmonella* bacteria such as STM1 provide a unique alternative in terms of heterologous antigen delivery and immune presentation. The STM1 mutant harbours an aroA gene mutation and is a well-characterised vaccine strain currently licensed for the prevention of *Salmonella* infection in livestock. Bachtiar et al., 2003 [3] have demonstrated its potential to deliver heterologous vaccine antigen expressed from plasmid and was found suitable as vaccine carrier for this purpose.

In this study, pHES and pMOhly1 vectors were used to express the influenza HA1 gene in STM1 for surface display and extracellular secretion of the antigen. The pHES vector equipped with a modified ShdA autotransporter gene derived from *S. Typhimurium* LT-2, which can translocate the rHA1 onto STM1 surface [5]. On the other hand, pMOhly1 vector encodes the necessary components of the *E. coli* α-haemolysin secretion system, which can secrete the rHA1 extracellularly [6].

The influenza PR-8 HA1 gene was successfully extracted from the virus and engineered into STM1 for surface display using the pHES vector and extracellular secretion using the
pMOhly1 vector. The expression studies have confirmed that the rHA1 expressed in pHES vector was successfully translocated onto the STM1 outer-surface membrane, with an optimal expression time of three hours post-induction. Furthermore, the rHA1 expressed in pMOhly1 vector was detected in overnight culture medium, indicating the successful secretion into extracellular space. Having confirmed expression of these STM1 vaccine clones, an animal experiment was carried out to assess the immunogenicity of these vaccines in Broiler chickens.

The results detailed in Chapter 5 indicated that the STM1 expressing the HA1 in the pHES vector failed to induce any detectable immune responses, which is probably attributed to its “one-off” antigen presentation due to the in vitro induced promoter. Furthermore, the pHES vaccine plasmid was undetectable immediately after immunisation, indicating the lack of plasmid retention in the chicken intestinal tract. It is possible that having the modified ShdA autotransporter gene expressed on the STM1 vaccine surface could severely influence the mechanisms to attach and colonise in vivo [386, 432], and the increase metabolic burden due to plasmid replication may have facilitated the spontaneous loss of plasmid [129]. Spontaneous loss of plasmid has been a challenge in the development of vaccine carriers; whilst the delivery of plasmid-borne S. Typhimurium vaccine carrier has been relatively successful, several reports have indicated a decreased efficacy due to plasmid instability [448-450]. Therefore, it is important to ensure the stability of the gene encoding the heterologous antigen to optimise the vaccine efficacy, and leading to the development of successful vaccine carriers.

The STM1 expressing HA1 using the pMOhly1 vector was also unable to induce any detectable humoral immune responses, including serum IgG, faecal IgA and virus-neutralising antibody responses. However, the vaccine successfully elicited a Th1-biased immune
response, indicated by the secretion of IFN-γ after being stimulated with either Pichia-expressed rHA1 or inactivated PR-8 virion. It was unfortunate that Th₂-type immune responses could not be examined in this study due to the unavailability of an anti-chicken IL-4 antibody for the ELISpot assay.

Although these vaccine vectors are as yet less than ideal for field application, the outstanding safety profiles and the ability to induce antigen-specific immune responses suggested that the development of Salmonella-vectored influenza vaccines could be achieved in the future by better expression of heterologous antigens from the selected Salmonella vector [379].

To improve the immunogenicity of the HA1 antigen delivered by STM1, the expression level and plasmid stability must be enhanced in order to progress to any commercial application. The use of plasmid-based antigen delivery system are a problem for two main reasons; they do not ensure the stability of antigen expression in the absence of selective pressure (antibiotic), and are open to the possibility of environmental contamination of residual bacterial flora through horizontal transmission of plasmids [4, 379]. However, the alternative plan would require the use of chromosomal integration coupled with an in vivo induced promoter, but such an expression system would decrease the level of antigen expression by a further step [379, 451, 452]. Therefore, further studies are required for selection of stronger promoters, as well, a well-characterised vaccine strain for chromosomal insertion.

Taken together, these results indicated that Pichia-expressed influenza rHA1 could induce not only virus-specific humoral immune responses but also stimulate Th₂-type cell-mediated immunity, which could be protective against influenza infections. The STM1 expressing secretory rHA1 using pMOhly1 vector successfully induced Th₁-biased cell-mediated immune responses, which is suggested to be important in delayed-type viral clearance [7, 447].
Although the results acquired in these studies were less than satisfactory, there were significant responses to the influenza antigen that suggested further investigation is required. It may be possible to induce a better well-balanced immune response against influenza virus by combining both the subunit rHA1 and the STM1 vaccine strain into a prime-boost vaccine scheme. Such a vaccine regime could ensure the elicitation of both humoral and cell-mediated immunity against influenza infections.

Moreover, it has been demonstrated in the study that the STM1 vaccine carrier can elicit Th$_1$-type immunity, hence it can potentially lead to help for the activation of CD8$^+$ T-lymphocyte responses against the heterologous protein. Ashraf et al., 2011 has demonstrated that after orally immunised mice with attenuated S. Typhimurium mutant carrying the influenza nucleoprotein, followed by three booster immunisations, the mice were completely protected against lethal influenza virus challenge. In addition, a Th$_1$-restricted immune response was elicited against influenza nucleoprotein [311]. Therefore, it is possible to use the STM1 vaccine carrier to deliver a conserved influenza viral protein such as the ion channel M2e or the NP, which might elicit a more universal immunity against the influenza infection.
Appendix 1

Lambda DNA digested with PstI run on a 1.5% agarose gel.

Image obtained from Fermentas Life Science Pty. Ltd., Japan
Appendix 2

Protein Marker, Broad Range (P7702), New England Biolabs, USA.
Appendix 3

Precision Plus Protein™ All Blue Standards (#161-0373), BioRad Laboratories, USA.
Appendix 4

ColorPlus™ Prestained Protein Marker (P7709), New England Biolabs, USA.
Appendix 5

Precision Plus Protein™ Dual Color Standards (#161-0374), BioRad Laboratories, USA.
Appendix 6

Precision Plus Protein™ Kaleidoscope™ Standards (#161-0375), BioRad Laboratories, USA.
Appendix 7

Individual IgG response against *Pichia*-expressed rHA1 in immunised mice, correlating to the aggregated data shown in Figure 3.12.
Appendix 8

ELISpot analysis of IL-4 and IFN-γ productions in stimulated T cells for individual mice, correlating to the aggregated data shown in Figure 3.13. and Figure 3.14.

Each bar is the illustration of averaged duplicates of an immunised mouse. Three mice were included in each cytokine production assay.
Appendix 9

Individual IgG response of pHES/STM1 and pHES/HA1/STM1 vaccinated chickens, correlating to the aggregated data shown in Figure 5.3.
Individual IgG response of pHES/STM1 vaccinated chickens against *Pichia*-expressed rHA1

Individual IgG response of pHES/HA1/STM1 vaccinated chickens against *Pichia*-expressed rHA1
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